JULY, 10, 1943

THE JOURNAL OF NUTRITION

VOLUME 26 NUMBER 1

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Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA 4, PA.
Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign

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FURTHER STUDIES ON THE SYMPTOMS OF MANGANESE DEFICIENCY IN THE RAT AND MOUSE [1]

MAURICE E. SHILS AND E. V. McCOLLUM
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FIVE FIGURES

(Received for publication January 2, 1943)

In the interval since 1931 when the first papers (Orent and McCollum, '31; Kemmerer, Elvehjem and Hart, '31) appeared with clear evidence for the necessity of manganese, this element has been found essential for normal reproduction, lactation, bone formation, growth and the activity of certain enzymes (Shils and McCollum, '42). Although all reports agree that manganese deficiency symptoms occur in rats and mice, they disagree as to the nature of these symptoms. Workers at Wisconsin have reported decreased growth and disturbances of the oestrous cycle resulting in sterility (Kemmerer, Elvehjem and Hart, '31; Waddell, Steenbock and Hart, '31; Skinner, Van Donk and Steenbock, '32; Boyer, Shaw and Phillips, '42). Orent and McCollum ('31) found no differences in oestrus or growth but, rather, poor reproduction with very high mortality of young and poor lactation performance. Daniels and Everson ('35), on the contrary, found lactation and oestrus both unaffected but observed high mortality of the young as a result of congenital debility.

The experiments reported here were initiated to gain further information on the effects of the deficiency in the hope that the apparently contradictory results of the preceding

[1] This work was aided by a grant from the Rockefeller Foundation.

THE JOURNAL OF NUTRITION, VOL. 26, NO. 1
JULY, 1943
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work might be clarified. This has been partly accomplished in that certain findings have been confirmed and extended. In addition, a new symptom of manganese deficiency in the rat has been observed.

DIETS

We have devised a solid basal diet low in manganese (0.2–0.3 μg. per gram) yet allowing excellent growth when supplemented with this element. It is easily made and consists of whole milk powder [2] 20.00 parts; cottonseed oil U.S.P. 11.68; casein and dried yeast extract [3] 16.00; sucrose 45.00; salts [4] 2.82; also, per kilo, percomorph oil 20 drops, choline chloride 200 mg., and thiamine chloride 1 mg. The drinking water was distilled and contained 1 ml. of 8% potassium iodide solution per liter. We designate this diet as diet 1 in this paper. Diet 21 is a modification in that pure vitamins [5] replaced the yeast extract; casein was fed at a level of 16.00. Diet and water were given ad libitum.

With the cottonseed oil and milk fat giving a high caloric value per gram the average manganese intake of an adult rat on this diet was less than 3 μg. per day. This is a somewhat higher level of intake than that on a diet consisting of whole milk, Fe, and Cu, such as used by the Wisconsin workers. However, it is known that a whole milk regime is not entirely adequate (Orent and McCollum, '32; Van Donk, Steenbock and Hart, '33; Richardson and Hogan, '40).

[2] Spray-dried by Roger process — Ward's.

[3] Yeast extract. Anheuser-Busch yeast was stirred for 3 hours in 50% alcohol (500 gm. yeast in 5 liters of alcohol), allowed to settle and filtered. 500 ml. extract were air-dried on 150 gm. casein. The casein was acid-washed and alcohol-extracted or washed.

[4] Salt mixture: K_2HPO_4 — 2250.00; $CaCO_3$ — 2340.00; $Fe(NO_3)_3 \cdot 9\,H_2O$ — 393.70; $MgSO_4 \cdot XH_2O$ — 450.00; NaCl — 900.00; Cu acetate — 15.00; $ZnSO_4 \cdot 7\,H_2O$ — 15.00. $Fe(NO_3)_3 \cdot 9\,H_2O$ was purified by recrystallizing $Fe(NO_3)_3 \cdot 9\,H_2O$ twice from 10: 1 nitric acid (Cleaves and Thompson, J. Res., Nat. Bur. Stand., *18*, 595, 1937), and once from water.

[5] The vitamins in diet 21 were per kilo: thiamine 2 mg.; riboflavin 3 mg.; pyridoxine 3 mg.; calcium pantothenate 5 mg.; inositol 3 mg.; choline chloride 200 mg. The synthetic vitamins used in this experiment were generously supplied by Merck and Company.

The control animals received manganous chloride ($MnCl_2 \cdot 4\,H_2O$) at a level of 0.18% in the diet for the first 6 months. The effect of manganese in producing rancidity became marked in hot weather and thereafter the manganese was omitted from the diet and added to the drinking water (100 mg. per liter). The regime with manganese is hereafter called diet 1 M.

The manganese content of the diet and various components was determined by a method essentially that of Skinner and Peterson ('30). The intensity of the permanganate color was determined in a Klett-Summerson photoelectric colorimeter using a glass absorption cell of 40 mm. Concentrations of manganese of 0.2 μg. per milliliter were detectable. By ashing relatively large quantities of diet (50–60 gm.), accurate determinations could be made. All solutions were brought to 50 ml. volume for colorimeter readings and the blank values were determined by adding 2–3 drops of 0.25% hydrogen peroxide solution directly to the solution in the colorimeter absorption cell to discharge the permanganate color (Rowland, '39).

RESULTS WITH THE RAT

Reproduction and lactation

Experiment 1 — Females. Forty stock females 3 weeks of age were paired according to litter into two groups. One group was placed on diet 1 alone; the other group received the basal diet plus manganese (diet 1 M). After 2 months, mating with fertile stock males was begun and continued periodically for over a year. The average number of litters obtained per animal was 3.4 with a minimum of 2 and a maximum of 7. There was a total of sixty-nine litters in the original deficient group; of these fifteen were born to seven animals given manganese after a long period on the deficient diet. The control group had seventy litters.

The pregnant females, when about to deliver their young, were placed in screenless reproduction cages with pans covered with filter paper clippings. These cages were examined twice daily and the young weighed when first seen.

Table 1 summarizes the reproduction history of the females in experiment 1.

Despite the fact that the viability records of the offspring of the control females did not equal those of stock rats, the difference between the viability performances of the young of the deficient and control groups is striking. Only four or 1.5% of the young born of deficient females were weaned and it is noteworthy that these four all occurred in one litter. All the

TABLE 1

Viability of offspring of females in experiment 1.

Diet	Number of					Number and per cent of	
	Females	Litters	Young found alive	Young found dead	Young kept with mother (A)	Young (A) dying within 48 hrs. of birth	(A) weaned
DEFICIENT FEMALES							
1	20	54	372	51	250	232 (93%)	4 [1] (1.5%)
1 M	7 [2]	15	54	21	44	19 (43%)	21 (48%)
CONTROL FEMALES							
1 M	20	70	424	66	331	122 [3] (37%)	185 (56%)

[1] All four were in one litter.

[2] These seven females were originally on diet no. 1 and after several pregnancies were placed on diet 1 M.

[3] Of the 122 that died 57 were offspring of two females.

other 246 young in fifty-three litters died, 93% of them in less than 48 hours. In only a very few young was there any visible evidence of milk.

When manganese was given to seven of the deficient females (which had lost all their young in nineteen litters while on the manganese-low diet), half of their young were weaned. The percentage viability of this group closely approximates that of the control females. It is evident that manganese is necessary for the production of viable young and that this aspect of manganese deficiency is reversible.

Despite the fact that their young did not survive, the behavior of the deficient females appeared normal. They built nests in most pregnancies and hovered over and carried their young normally. They suckled and raised foster stock and control young. These observations on behavior of the deficient mothers are at variance with the observations of Orent and McCollum ('31). It was noted in some instances that the mother lost interest in her young, but this apparently occurred only when they did not suckle.

The failure of many of the young of the control animals to live cannot be explained at present. It is of interest to note, however, that the results of the individual females varied widely. Seven females weaned the young of all their litters and only two lost all their young; two of the twenty females were responsible for 47% of the non-viable young. That the results were not due to a borderline deficiency of the B complex is indicated by the fact that increasing the yeast extract or synthetic B vitamin content of the diet had no effect on the viability of the young of either group.

Experiment 1 — Males. Twenty-four males were placed on diet 1 at weaning and sixteen littermates of the same sex were placed on diet 2 M at weaning. No significant differences in growth, sperm motility, or testicular weight were noted over a period of 14 months.

Exchange of young

The results obtained upon exchanging and transferring young were similar to those of Daniels and Everson ('35). Eighty-three deficient young from seventeen litters were transferred to stock or control lactating females within 24 hours after birth but only three of these lived to 21 days. When eighty-four stock and control young from twenty-three litters were given to manganese-deficient mothers, forty-nine lived 21 days. Thus the deficient mothers were able to raise foster young while their own young died. In most instances where there was failure of a deficient female to suckle foster

young, the failure occurred with first litters. With subsequent litters the foster young were weaned in almost all cases.

Fertility and oestrous cycle

Observations were made on fertility and oestrous cycle of the females of both groups throughout experiment 1. For the first 10 months on the diets both groups exhibited a normal oestrous cycle and reproduction. Thereafter a prolongation of the oestrous cycles was frequently but not always found, with the affected females remaining in the postoestrum or anoestrum stages of the cycle for weeks. Mating did not occur. There appeared to be no significant difference between the deficient and control groups.

Reversibility of manganese deficiency in females

The symptoms of manganese deficiency in females can be overcome by the administration of this element even after 428 days on the deficient diet. As indicated in table 1, upon manganese administration the percentage of young weaned changed from 1.5 to 48, the latter figure approximating that of the females of the control group.

Development of manganese deficiency in adult females (experiment 1 A)

Manganese deficiency can be produced in adult females. In this experiment eleven stock females 5 to 6 months of age were used after five had been tested for fertility on stock diet.

When these females were mated, maintained on stock diet until near the end of pregnancy, and then placed on the deficient diet, the young that were born appeared normal. However, when these young were weaned and divided into two groups, one group on the deficient diet and the other receiving manganese, marked growth differences became apparent, particularly with the males. Continued mating of the adult females on the deficient diet resulted in increasingly poor viability performance of their offspring. Many of these young

showed symptoms of ataxia, poor equilibrium, and weakness in the third week of life, and were markedly underweight. In still later litters all of the young died soon after birth.

Development of young

Almost all of the young of the deficient females in experiment 1 died soon after birth. No definite pathological changes were observed [6] in these young. The deficiency presumably results in an acute biochemical lesion in some system or systems of fundamental importance after birth. There appeared to be no significant differences between the birth weights of the deficient and control young.

The few young of the deficient females of experiment 1 which lived as long as 21 days were subnormal in weight and in addition exhibited an ataxia, incoordination and loss of equilibrium. The offspring of deficient females in other experiments which survived long enough almost invariably developed the same symptoms. These were apparent by the end of the second week of life and were characterized by rotational movements. Occasionally the affected animal walked in a straightforward manner but soon fell over on its side or back, righting itself with difficulty. The loss of equilibrium was marked and included loss of the righting reaction. Usually the animals were quite active. The symptoms increased in severity to about the eighteenth day, whereupon the animal either died or else recovered to a great extent; however a noticeable failure of the righting reaction not infrequently persisted.

This condition was best produced by developing the deficiency in mature females. The young of the second litter usually showed the symptoms. It is interesting to note that Caskey and Norris ('40) observed an ataxia in the offspring of manganese-deficient hens.

Skeletal development

Abnormal bone development in manganese-deficient rats has not hitherto been demonstrated with certainty, although

[6] Follis, R., Jr. and Shils, M. E., to be published.

the possibility of its occurrence is indicated by the report of Barnes, Sperling and Maynard ('41). It is well-known that in manganese-deficient chicks skeletal abnormality is the outstanding symptom.

Our preliminary results indicate that the surviving young of markedly deficient females develop skeletal abnormalities. The gross effect is a shortening and bowing of the forelegs. The pathological changes will be described elsewhere in detail.[6]

Growth

We have found that manganese is essential for optimum growth, thus confirming the observation of Boyer, Shaw and Phillips ('42). This was demonstrated in the offspring of depleted mothers (experiments 1, 1 A and 4) and also in first-generation rats on modifications of diet 1 (experiments 7 and 8).

In experiment 1 no decreased growth was noted in the deficient rats which were on diet 1 since weaning. However, in the few surviving offspring of this group, marked decreases in growth were noted (fig. 1). In experiment 1 A as well as in experiment 4 decreased growth was marked in the offspring of adult females placed on the deficient diet late in pregnancy.

Experiment 4. In this experiment stock females were placed upon the manganese-low diet (diet 1) during the last week of pregnancy and maintained on it during lactation. At 21 days the young which were all of good weight and appearance were divided into two groups as littermate pairs; one group received the manganese-low diet, the other the same diet plus manganese. Growth records on twenty males and sixteen females are shown in figure 2. The differences in growth between the deficient and the control animals are obvious and are especially marked with the males. The decreased growth of the deficient males is associated with sterility and poor appearance of the fur.

Just as the reproduction symptoms of deficiency in the female can be reversed by administering manganese, so the

decreased growth of deficient males and females can be reversed by giving manganese even after 38 weeks on the deficient diet (fig. 3).

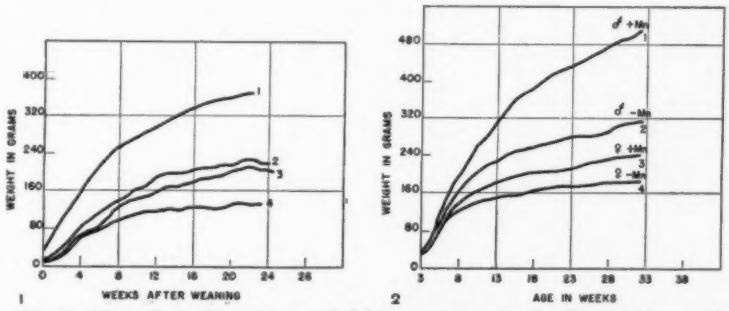

Fig. 1 The effect of manganese deficiency on growth of offspring (expt. 1). Curve 1 illustrates the growth performance of a typical male offspring of a control female on diet 1 M. Curves 2 and 3 are for the males and curve 4 for the female which were three of the only four deficient young to be raised to weaning by a deficient mother (table 1). The deficient rats also showed marked incoordination.

Fig. 2 The effect of manganese deficiency on growth (expt. 4). All the rats used in these experiments were the offspring of females started on the deficient diet during the last 4 to 7 days of pregnancy. At weaning the littermates whose weights are illustrated were paired, one-half being maintained on the deficient diet of the mother (diet 1) and the other half on the same diet supplemented with manganese (diet 1 M).

Curves 1 and 3 are, respectively, based on the average weights of ten males and eight females receiving manganese; curves 2 and 4 are those of like numbers of deficient rats. The differences are particularly marked with the males.

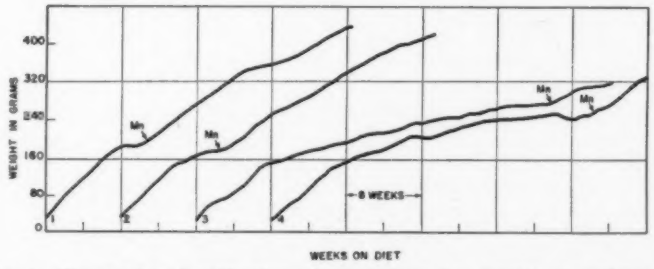

Fig. 3 The need and utilization of manganese for growth at various ages (expt. 4 C). Growth curves are given for four male littermate rats which were the offspring of a mother placed on the manganese-deficient diet a week before delivery. These young were continued on the deficient diet until manganese was added at the time indicated by an arrow.

These growth results indicate that manganese is needed by the rat not only during reproduction and lactation, but also during the period of rapid growth, and that it can probably be utilized and possibly is necessary at all stages of life.

Sterility in deficient males

The occurrence of sterility and testicular atrophy in manganese-deficient males was first noted by Orent and McCollum ('31) and has been confirmed by others. We have likewise been able to produce sterility and testicular atrophy in the great majority of the males of experiment 4, that is, in males whose mothers were on the deficient diets during pregnancy. No testicular atrophy was noted in the males in experiment 1, although there were histological changes.[7]

Effects on growth of changing the Ca and P levels in the manganese-low diets (experiments 7 and 8). It is well known that increasing the Ca or P levels in the diet of chicks on low manganese diets accentuates the appearance of perosis (Wilgus and Patton, '39). In addition, the Ca : P ratio of the manganese-low diet used by Orent and McCollum ('31) was calculated at 0.46; whereas the Ca : P ratio in our diet was 1.4.

In order to determine (a) whether more severe symptoms could be induced by altering the Ca : P ratio and (b) whether the differences in Ca : P ratio between the two manganese-low diets of Orent and McCollum ('31) and that described here

[7] The modifications from Baldwin ('35) were, briefly: The use of a glycine buffer pH 9.5 instead of NaOH with which to make the liver brei. A few milliliters of the buffer were used to grind liver to a paste and then the rest of 25 ml. of the buffer used, together with distilled water to bring volume to 100 ml. The flasks were shaken 7–10 minutes and filtered through filter paper on a Buchner funnel. This procedure was found more desirable since the brei did not become gelatinous, and allowed more certain adjustment of pH: 2.5 ml. brei, 0.5 glycine buffer, and sufficient water or manganese solution added to bring volume to 5.1 ml. Suitable blanks were used. The substrate was 2.0 ml. of 5% arginine carbonate (Eastman) or arginine monohydrochloride (Eastman). The monohydrochloride solution was made up with sufficient NaOH to neutralize the HCl calculated to be present with the arginine. The reaction was stopped after 30 minutes at 28°C. and the tubes heated in boiling water. The contents were diluted to 25 ml. without filtering and suitable aliquots used for urea determinations by the method of Krebs and Henseleit ('32) using a suspension of Squibb's urease tablets.

could account for the differences in results the following experiments were performed.

Experiment 7. The P content of diet no. 1 was increased by the addition of K_2HPO_4 until the Ca : P ratio was 0.46 (that of Orent and McCollum's diet, '31). Four females and six males (stock weanlings) were placed on the manganese-deficient "high" P diet (diet 7) and five females and five males (littermates) were placed on the same diet but received manganese in the drinking water.

The growth and reproductive behavior of the females were the same as had been observed on the diets without added P. The deficient females consistently lost their young, whereas the controls weaned many of theirs. There were no significant differences in growth.

With the males, however, definite differences in growth were observed (fig. 4) in all instances. After 36 weeks on the deficient diet, the animals responded to manganese (fig. 4).

Experiment 8. The content of the original diet 21 was increased by the addition of $Ca_3(PO_4)_2$ so that the Ca was raised from 0.614% to 1.5% and the P from 0.46% to 0.9%, with a change in the Ca : P ratio from 1.4 to 1.66. Two littermate groups of six rats each were used, one receiving the deficient diet (diet 28) and the other receiving diet 28 plus manganese. Here again very significant differences were noted in growth between the two groups. The deficient males responded to manganese after 5 months (fig. 5).

Inasmuch as no growth differences were noted in males on the original diet, the differences in experiments 7 and 8 seem ascribable to the increased amounts of P alone and Ca and P together in the diets. As found in the work with chicks (Wilgus and Patton, '39), the increased amounts of Ca and P intensify the symptoms of manganese deficiency, and in all probability act in a similar fashion by rendering less available the manganese in the diet.

RESULTS WITH MICE

Mice were placed on the experimental diets (1 and 1 M) soon after weaning. Reproductive results indicate that the

mice behaved like the rats. Four females on the deficient diet had a total of ten litters with sixty-one young, all but six of which died within 48 hours. None lived more than 3 days. Five control females on the same diet receiving manganese in the drinking water had fourteen litters with ninety-five young, thirty-three of which were weaned.

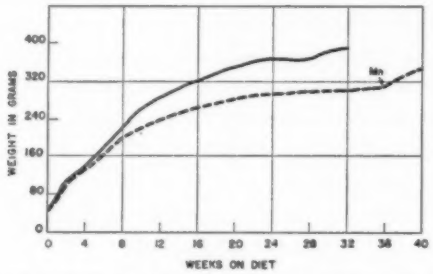

Fig. 4 The need and utilization of manganese for growth (expt. 7). Stock males were placed at weaning on manganese-deficient diet 7 and as the broken-line curve (average for six rats) indicates, did not grow as well as littermate controls receiving the same diet plus manganese (solid line, average for five rats). The deficient rats were able to respond to manganese after 36 weeks. Diet 7 had an increased phosphorus content over that of diet 1, on which no decreased growth was noted.

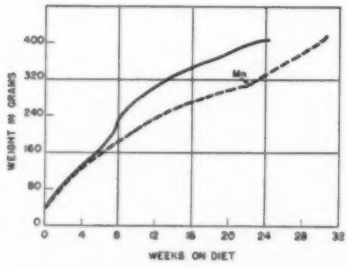

Fig. 5 The need and utilization of manganese for growth (expt. 8). Stock males were placed on deficient diet 28 at weaning (broken line) and showed decreased growth in comparison with littermate controls receiving manganese. The deficient animals gave a marked response to manganese after 22 weeks. Curves are averages for six rats. Diet 28 had a higher Ca and P content than diet 1.

ARGINASE ACTIVITY

The reports of Edlbacher and Pinosch ('37) and of Richards and Hellerman ('40) on the activation of manganese of liver arginase preparations led naturally to the measurement of arginase activity in manganese-deficient rats. Arginase has assumed prominence since Krebs and Henseleit ('32) demonstrated its role in the formation of urea.

The procedure used for the determination of the activity of the enzyme was a modification of the method of Baldwin ('35).[7] One gram samples were used in all instances to make the liver brei and only one concentration of this brei was used in the determination of arginase activity. All results are expressed as milligrams of urea formed in 30 minutes by 1 mg. dry weight of liver. The moisture and fat contents of the livers of the deficient and control rats were the same.

That the method gave reproducible results is indicated by numerous determinations of enzyme activity on three samples, each done in duplicate, from the same liver. In no instance was the variation more than 3½% from the average.

Results

Arginase activity has been determined on fourteen deficient and thirteen control rats and on several stock animals; their ages varied from 10 to 16 months. The results have been quite consistent for each group and representative figures are given in table 2.

It is evident (a) that there is a decrease of approximately 50% in the arginase activity in the livers of the deficient rats; (b) that the addition of manganese increases the activity in both groups and raises the activity in the deficient group to that of the controls, this latter point being evidence that the formation of the protein component of the enzyme is not affected by deficiency of manganese; and (c) arginase activity in the females is appreciably lower than that in the males in both groups.[8]

[8] Others (see Lightbody, '38) have previously noted this sex difference. However, Lightbody ('38) found in his series of rats that by the 200th day of life the sex difference had disappeared; in our study the difference was still marked in rats 480 days old.

In several instances where arginine monohydrochloride rather than arginine carbonate was used as substrate, a similar decrease in activity of the liver preparation from the deficient rats occurred. However, it is noteworthy that with arginine monohydrochloride as substrate the addition of manganese, although it increased the activity in both groups, did not restore that of the deficient group to the level characteristic of the control rats. These results, considered without those with arginine carbonate as the substrate, might have led to the conclusion that the enzyme concentration as well as

TABLE 2

Arginase activity in the liver of manganese-deficient and control rats. (5% arginine carbonate as substrate.)

CONTROL RAT NO.	ARGINASE ACTIVITY [1]		DEFICIENT RAT NO.	ARGINASE ACTIVITY [1]	
	No added manganese	60 μg. manganese added		No added manganese	60 μg. manganese added
21 B ♂	0.930	1.826	21 A ♂	0.642	1.832
22 B ♂	1.230	1.687	22 A ♂	0.661	1.770
29 B ♂	1.016	1.593	29 A ♂	0.548	1.524
2 D–22 B ♂	1.182	1.509	2 D–22 A ♂	0.548	1.433
15 B ♀	0.896	1.684	1 A ♀	0.406	1.331
Average	1.051	1.660	Average	0.561	1.558

[1] Expressed as milligrams uren formed in one-half hour by 1 mg. dry weight liver at 28°C.

the activity was decreased in the deficient livers. Boyer, Shaw and Phillips ('42) used arginine monohydrochloride and likewise found that manganese did not restore the liver arginase activity of manganese-deficient rats to that of the controls. They concluded that the decreased activity "represented differences of enzyme concentration and not of activation." Our results with the carbonate do not support this conclusion; however, we cannot account for the differences between the two substrates, except perhaps as an effect of CO_3^- or Ce^-.

The finding of a decreased liver arginase activity in vitro raised the question as to whether decreased arginase activity

in vivo could account for the symptoms observed in the deficient animals. Studies were initiated on the growth and symptomatology of young manganese-deficient rats given ammonium citrate at a level of 5% in the diet. Since arginase is concerned with the formation of urea (Krebs and Henseleit, '32), it might be expected that an increased burden on the urea-forming system might result in an accentuation of symptoms. In a preliminary experiment, eighteen young born of partially deficient females were divided into three groups. One group received the manganese-low diet, the second group the manganese-low diet plus 5% ammonium citrate, and the third group the manganese-low diet plus manganese plus 5% ammonium citrate. In 9 months no significant differences were observed between the deficient animals receiving the ammonium citrate and those not receiving it, although both of these groups did not grow as well as the group receiving manganese. This supports the observation (Boyer, Shaw and Phillips, '42) that there is no difference in nitrogen excretion in manganese deficiency.

A decreased activity of arginase found in vitro may not occur in vivo or, if it does, it may not affect the animal adversely until a still greater decrease occurs. More work is needed in an attempt to link decreased arginase activity to the symptoms of manganese deficiency. The finding of Lightbody ('38) is of interest in this connection in that he found a very great increase in arginase activity in the livers of rats soon after birth, that is, at the time when most of our manganese-deficient young died.

DISCUSSION

The findings reported here, together with those of other workers already mentioned, indicate that the symptoms of manganese deficiency constitute a complex both interesting and far from understood. The fact that the congenitally debilitated young show no histologic abnormalities is an indication of an acute "biochemical" lesion. The evidences of incoordination and poor equilibrium implicate manganese in

the functioning of the nervous system. The bone changes indicate that in rats as well as chickens manganese plays a role in skeletal formation and Ca and P metabolism.

The conflicting reports in the literature are challenging and we shall attempt to reconcile at least some of them.

Female reproductive effects. The differences between the Hopkins and Wisconsin workers may indeed be caused by the differences in diets. Milk as the main component is lower in manganese than the more complex solid diet used by us. Consequently the effect on the oestrous cycle may be the result of a more acute deficiency.

Since the literature on manganese deficiency appears so contradictory we think it worth while to summarize the points of agreement.

(a) Our finding and that of Boyer et al. ('42) that on a given diet rats and mice behave alike indicate no species differences and are further support for the belief that differences in observations in various laboratories are due to the variations in manganese levels of the diets.

(b) There is agreement that manganese deficiency (probably of a particular quantitative degree) results in congenital debility of young (our results; part of Orent and McCollum ('31) and Daniels and Everson, '35).

(c) It is agreed that manganese is essential for growth of rats and mice (our findings; Kemmerer, Elvehjem and Hart, '31; Boyer et al., '42).

(d) All work with rats is in agreement that the deficiency results in testicular atrophy and sterility.

(e) There is a decrease in arginase activity in vitro. We agree with Boyer et al. ('42) that there is decreased activity but not that there is decreased formation and concentration of the protein part of the enzyme.

(f) Our findings on bone changes are in agreement with the findings of manganese-deficient chicks.

(g) Our finding of a need for manganese for normal nervous system functioning is in agreement with observations made on manganese-deficient chicks.

There are some differences not so readily explained. The inability of the deficient females in the Orent and McCollum experiment ('31), in eight out of ten cases, to suckle stock or control young is at variance with our findings and those of Daniels and Everson ('35). Likewise we were unable to note any marked difference in the behavior of the deficient females toward their young.

From all this we conclude that it is apparently possible to develop three distinct stages of manganese deficiency in the female. In the least severe stage, the deficient female gives birth to viable young which manifest symptoms of incoordination, paralysis and poor equilibrium that are most marked in the third week of life. In the second and more severe stage, non-viable young are born which die very shortly after birth. In the third and most severe condition, the oestrous cycle is disturbed to the point where sterility results.

SUMMARY

1. A solid basal diet has been devised which is very low in manganese and which when supplemented with this element allows much better growth than any manganese-low diet heretofore devised.

2. Deficiency of manganese in first generation female rats started on the manganese-low diet at weaning resulted in the production of non-viable young. The deficient females were capable of raising foster stock or control young, although their own offspring died. No abnormalities were noted in the behavior of the deficient females towards their young. No disturbance in oestrous cycle due to manganese was noted in first generation females. Deficient mice likewise gave birth to non-viable young.

3. No decrease in growth was noted in the deficient rats of the first generation on the basal diet. However, the deficient offspring, particularly the males, of stock females placed on the deficient diet late in pregnancy, were inferior in growth to littermates given manganese. Likewise, increasing the calcium and phosphorus contents of the basal diet resulted in a marked

decrease in growth of first generation males. Manganese is a factor necessary for optimum growth. The deficient males were sterile.

4. A hitherto undescribed symptom of manganese-deficiency was observed in young rats, characterized by loss of both equilibrium and coordination.

5. The symptoms of manganese deficiency, namely, production of inferior young and poor growth of the female and sterility and poor growth of the male, are reversible by administration of manganese.

6. A marked decrease in arginase activity of the livers of the deficient rats has been noted.

7. The observations of various authors on the effects of manganese deficiency are discussed in connection with these results.

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THE ROLE OF DIETARY PROTEIN IN HEMOGLOBIN FORMATION [1]

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(Received for publication January 4, 1943)

The fact that hemoglobin is protein in nature led to the early belief that an adequate amount of dietary protein is essential for its formation. Indeed, Voit (see Jackson, '25) believed that the hemoglobin content of the blood varies to some extent with the protein content of the diet, and Rubner ('19) suggested that much of the widespread anemia in Germany following World War I was due in part to an inadequate intake of protein. Pernicious anemia was once believed to be related to "faulty digestion" (Fenwick, 1877), a view which prompted Minot and Murphy ('26) to study the effect of the treatment of the disease with a protein and iron-rich (liver) diet. The classical work of Castle ('29) may be interpreted as a further suggestion of a possible derangement in the metabolism of some protein derivative in pernicious anemia, a view which cannot be disregarded today because of the alleged peptide nature of the anti-pernicious anemia factor.

[1] Aided by a grant from the Committee on Research, American Association for the Advancement of Science.

A portion of the data in this paper is taken from a dissertation presented by Aline Underhill Orten in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1937.

Preliminary reports were made before the American Society of Biological Chemists, Toronto, 1939, and the American Institute of Nutrition, New Orleans, 1940.

Experimental studies (Jencks, '22; McCay, '28) on rats and dogs with hemorrhagic anemia have given some indication that there is a relation between dietary protein and hemoglobin formation. The rate of hemoglobin regeneration after hemorrhage was found to be more rapid in animals allowed an adequate protein intake. However, the possible importance of concomitant variations in the intake of calories and minerals was not determined in these investigations. Studies in this laboratory (Orten and Smith, '37; Orten and Orten, '39) have demonstrated that a mild chronic anemia develops in rats fed a low-protein diet in contrast to a normal blood picture in controls given an adequate dietary protein intake. The work of Whipple and his collaborators (Hahn and Whipple, '39) has shown that in dogs maintained in a constant state of anemia from hemorrhage, more hemoglobin is formed when the diet contains an adequate amount of protein than when the protein content is low, even though the iron intake is adequate. Further evidence that dietary protein is involved in hemoglobin production is found in the observation (Bethell, '36) that the anemia of pregnancy in human subjects frequently may be due to a lack of adequate dietary protein and may be benefited by increasing the intake of protein.

The present investigation was designed to study further the relation of the energy intake and of iron to the chronic anemia produced in rats by the feeding of a diet low in protein.

EXPERIMENTAL

Twenty-one day old weanling female rats of the Connecticut Agricultural Experiment Station strain, weighing from 40–50 gm., were used. They were housed in individual cages having a wide mesh screen bottom. Five groups of animals were studied. Group 1, "adequate protein," received a synthetic diet consisting of 18% lactalbumin,[2] 10% sucrose, 41% white dextrin, 27% hydrogenated vegetable oil, and 4% salt mixture (Wesson, '32). The food consumption was recorded

[2] "Labco" lactalbumin, The Borden Company, Bainbridge, N. Y.

every 4 days; the average daily food intake for each rat of this group over a period of 76 days was found to be 8.8 gm. Group 2 received a "low-protein" diet having the same composition as the "adequate" diet except that it contained 3.5% lactalbumin and 55.5% dextrin. The average daily food consumption of rats of this group was found to be 4.9 gm.

Group 3, a "calorie weight" control group, received the same allowance of calories daily as was consumed by the "low-protein" group of rats (group 2), but a daily protein intake equal to that of the "adequate protein" rats (group 1) of the same weight. For example, during one typical 4-day period the amount of food given the "calorie-weight" controls was 19.2 gm., the average amount consumed by the low protein (group 2) animals during the same age-period. The average body weight of the "calorie-weight" animals at this time was 76 gm. When the adequate protein animals (group 1) weighed 76 gm. they consumed an amount of diet in 4 days which contained 5.9 gm. of protein. Therefore, the "calorie-weight" animals were fed a diet during this particular period so prepared that 19.2 gm. contained 5.9 gm. of lactalbumin (30.9% of the diet). The diet, of course, contained less dextrin but exactly the same amounts of fat, sucrose, and salt mixture as 19.2 gm. of the low-protein diet. Obviously, it was necessary to reconstruct this diet every 4 days. During the entire experiment the percentage of protein varied from 20.6 to 35.2, with an average of 31.0%.

Also, an "inanition control" group of rats (group 4) was studied by the paired feeding technique. These animals were paired with litter mates of the low-protein group and received the same daily amount of calories (and minerals) as did their low-protein pairs; but they received more protein since they were fed iso-caloric amounts of the "adequate protein" diet, containing 18% lactalbumin.

In order to further study the possible effect of calories, a diet of increased caloric value but of low protein content was fed to a small group of animals, "high-calorie" (group 5). These rats were given the average amount of low-protein diet

consumed by the rats of group 2 during each 4-day feeding period and in addition fat and sucrose in such amounts that the total number of calories ingested would be equal to that consumed by the "adequate protein" animals of group 1 during the same period. The fat and sucrose were added in the same proportions in which they appeared in all of the other diets.

All animals received the following vitamin supplements: 200 mg. of a rice polish extract [3] and 100 mg. liver extract [4] no. 343 daily, and 2 drops of cod liver oil [5] concentrate twice weekly. In preliminary experiments these quantities of vitamins were shown to be adequate in half these amounts; nevertheless full amounts were fed to further insure against a possible inadequacy of vitamins due to any altered requirement brought about by the protein deficiency. If an animal did not voluntarily consume all of the supplement, the remainder was force-fed.

At the end of the period of 76 days just described, the anemic, low-protein rats, together with additional similar animals started at a later date, were divided into three subgroups. One (group 2 A) was continued on the same low-protein diet. A second sub-group (2 B) was given a diet supplying the same average daily intake of calories as the "low-protein" group of rats had been consuming, but the protein content was increased to provide a daily protein intake equal to the average daily protein consumption of the "adequate protein" control rats (group 1). This made a diet containing 40% protein. The third sub-group (2 C) was continued on the low-protein diet, but was given 1.0 mg. iron as $FeSO_4$ daily as a supplement. This was added in solution to the vitamin supplements. Twelve of the group of "adequate protein" rats (group 1) were continued as normal controls on their previous diet.

[3] Ryzamin B, obtained from Burroughs Welcome and Company, Tuckahoe, N. Y.

[4] Appreciation is expressed to Dr. C. P. Rhodehamel of the Eli Lilly Company for a generous supply of liver extract.

[5] White's.

Hemoglobin determinations were made on blood obtained from a tail vein of all animals on the seventy-sixth day of the experiment and at the intervals thereafter indicated in the tables. A modified acid-hematin method was used and all readings were made in a photoelectric colorimeter calibrated with bloods on which the hemoglobin content had been determined by the oxygen capacity method.[6] Erythrocyte and reticulocyte counts were determined on the seventy-sixth day of the experiment. Bureau of standards certified pipettes and counting chambers were used in making the red cell counts. The reticulocyte counts were made on permanent smears stained with brilliant cresyl blue and counterstained with Wright's stain (Orten and Smith, '34). Body weights were determined weekly.

RESULTS AND DISCUSSION

The averaged results of the blood studies on the five groups of rats after 76 days on the experiment are given in table 1.

TABLE 1

Hematological findings in control rats and in rats fed a diet low in protein[1]

GROUP	DIET	NO. OF RATS	HEMOGLOBIN	ERYTHROCYTES	RETICULOCYTES
			gm./100 cc.	*M/cmm.*	%
1	Adequate-protein	15	15.8±0.7	8.1±0.6	2.2±0.2
2	Low-protein	26	11.5±0.9	8.1±0.9	7.3±3.5
3	Calorie-weight	8	15.7±0.6	8.8±0.4	2.2±0.6
4	Inanition control	8	14.8±0.7	8.8±0.2	2.2±0.5
5	High-calorie	4	9.9±2.1	7.5±1.4	16.1

[1] Group average values, together with standard deviations, obtained after the animals had been fed the various diets for 76 days.

The "adequate-protein" rats (group 1) showed hemoglobin values, erythrocyte counts, and reticulocyte counts which are normal for rats of that age (Orten and Smith, '37). The "low-protein animals" (group 2), on the other hand, consistently showed a mild chronic anemia, characterized by a distinctly

[6] Appreciation is expressed to Dr. Victor Schelling of Henry Ford Hospital for supplying bloods of known oxygen capacity content.

sub-normal hemoglobin content of the blood, a normal erythrocyte count, but an elevated reticulocyte count. The distinct reticulocytosis confirms the existence of a mild anemia in the animals fed the low-protein diet and, furthermore, indicates that there is a compensatory attempt by the bone marrow to increase the erythrocyte output. The "calorie-weight" control rats (group 3), given an abundance of protein but a restricted intake of calories had an essentially normal blood picture. This observation is convincing evidence that the anemia of the low-protein animals is due to an insufficient consumption of protein rather than to an inadequate intake of calories. This statement is further borne out by data obtained from both the "inanition controls" (group 4) and the "high calorie" rats (group 5). The daily caloric and mineral intakes of the inanition controls were the same as those of the low-protein rats (group 2) but the protein intake was greater, since the diet contained 18% protein. As is evident from the data in table 1, the inanition controls (group 4) showed an almost normal blood picture. It is to be noted, however, that the average hemoglobin level, while in the normal range, is less than that of the calorie-weight rats (group 3). This is undoubtedly due to the fact that the inanition control rats received less protein daily than did the calorie-weight animals, since the daily amounts of all other dietary constituents ingested by the two groups of rats were identical.

The data obtained on the "high-calorie" rats (group 5), given an adequate intake of calories but a restricted protein intake, demonstrate the inability of an increased consumption of calories to improve the hematological picture in rats fed the low-protein diet. In fact the animals of this group were even more severely anemic than were the "low-protein" rats, the hemoglobin and erythrocyte values being lower and the reticulocyte counts considerably higher. The increased severity of the anemia in this group may be due to the increased amount of fat in the diet, since a high fat diet apparently increases the rate of erythrocyte destruction (Loewy, Freeman, Marchello, and Johnson, '43). It was possible to

study only a few animals in this group because of the difficulties entailed in force-feeding the animals a relatively large amount of the diet each day.

The animals given the adequate protein diet showed an excellent rate of growth, attaining an average weight of 235 gm. at 97 days of age, which is greater than that of normal stock female rats of the strain employed (Smith and Bing, '28). The rats fed the low level of lactalbumin, on the other hand, grew very little, the average body weight being only 82 gm. at the end of the experimental period. The average weights of the "calorie-weight" control group (147 gm.) and the "inanition control" group (135 gm.) were greater than that of the low protein rats, showing that some of the added protein was undoubtedly being used to form tissue protein as well as hemoglobin. However, since the increase in body weight toward a normal value for rats of this age was relatively small in comparison with the increase of the hemoglobin level to practically normal, this observation may be interpreted as supporting the view (Whipple, '42) that hemoglobin synthesis occurs preferentially over body tissue protein formation. In contrast, the average body weight of the "high calorie" control rats (68 gm.), was even less than that of the "low-protein" group, despite the increased amount of calories ingested.

The results obtained from the hemoglobin determinations on the adequate-protein rats and various sub-groups of low-protein animals continued on the experiment are given in table 2. It is evident that the adequate protein control animals continued to maintain a normal amount of hemoglobin in the blood (Orten and Smith, '34) whereas the anemia persisted in the rats (group 2 A) continued on the low-protein ration and it became even more pronounced by the end of the experiment. The anemia has been found to increase progressively in severity in animals maintained on this regime for nearly 1 year.

When the low-protein rats were given the increased amount of protein (group 2 B), a remission of the anemia promptly

occurred. This took place in spite of an unaltered allowance of calories, iron, and other dietary constituents. The hemoglobin level in the blood of these rats became normal within 4 weeks after protein realimentation. This was accompanied by an average increase in body weight of 25 gm. On the other hand, increasing the iron intake of animals maintained on the low-protein diet (group 2 C), resulted in no consistent improvement. There was a slight transient rise in the hemoglobin level but at the end of the experiment the hemoglobin

TABLE 2

Hemoglobin content of the blood of control rats and of rats fed a diet low in protein supplemented with protein or iron [1]

GROUP	DIET	NO. OF RATS	HEMOGLOBIN — GM. PER 100 CC.					
			Initial	2 wks.	4 wks.	8 wks.	12 wks.	16 wks.
1	Adequate-protein (cont'd.)	12	16.5±0.3	16.6±0.3	16.3±0.2	16.7±0.2	16.1±0.2	16.2±0.2
2 A	Low-protein (cont'd.)	10	12.2±1.2	12.1±1.4	12.1±1.5	11.7±1.0	11.3± 1.0	11.6±1.0
2 B	Low-protein + protein	12	11.5±1.7	14.9±0.2	16.1±0.2	16.1±1.0	15.4±1.0	15.5±1.0
2 C	Low-protein + iron	8	12.0±1.3	12.5±1.7	12.3±1.9	12.6±1.8	12.8±1.9	11.5±1.9

[1] Group average hemoglobin values, together with the standard deviations.

level of this group of rats was no greater than that of those given no added iron. The inability of added iron to increase to normal values the hemoglobin content of the blood of any of the low-protein animals indicates that a lack of iron is not primarily involved in the anemia of these rats. Also, since an unpurified iron salt was used there is no indication that the "trace elements," such as copper or cobalt, are involved. There was no beneficial effect of the supplemental iron on growth.

The foregoing experiments taken as a whole offer strong evidence that an adequate quantity of dietary protein is essential for normal hemoglobin formation in the rat and that the caloric intake is of minor importance. The mild chronic anemia occurred in all animals fed the low-protein (lactalbumin) diet, whereas a normal blood picture was found in rats given the same amount of diet containing an adequate quantity of protein but a restricted amount of calories. Furthermore, the chronic "low-protein anemia" developed in spite of an increased intake of calories. Further evidence that the observed anemia is due primarily to the low-protein intake was obtained in the group of "low-protein" rats realimented with an adequate amount of protein but with no change in the intake of calories, minerals, or other dietary constituents. A prompt remission of the anemia resulted in these animals.

The results obtained in the present experiment obviously give no insight into the mechanism involved in the development of the anemia in rats fed the low-protein diet. One logical explanation would be that a low protein intake simply decreases the formation of the body protein, hemoglobin. However, it is equally possible that the low protein intake may alter the absorption, retention, or utilization of some other substance, or substances, which is essential for normal hematopoiesis. Possible support to such an interpretation is the fact that rats fed a low-protein diet show diminished amounts of riboflavin in the liver, even though additional riboflavin is given (Sarett and Perlzweig, '43), and this vitamin appears to have a distinct effect on hematopoiesis in the hemorrhagic anemia of dogs (György, Robscheit-Robbins, and Whipple, '38). This question merits further careful consideration.

SUMMARY

The administration of a diet low in protein (lactalbumin) but adequate in all other known respects produces a mild chronic anemia in rats.

The "low-protein anemia" may be prevented or cured by the allowance of an adequate protein intake without an

alteration in the amounts of calories, minerals, or vitamins consumed.

Increasing the intake of either calories or iron has no consistent beneficial effect on hemoglobin formation in the low-protein animals.

These observations warrant the conclusion that an adequate intake of dietary protein is essential for normal hemoglobin formation in the rat.

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THE EFFECT OF CONCENTRATION ON THE ABSORPTION OF VITAMIN A [1]

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(Received for publication January 11, 1943)

There is considerable evidence that vitamin A is readily absorbed from the gastrointestinal tract. According to Clausen ('33) vitamin A in the blood reaches a maximum 3 hours after its oral administration, which would indicate either that a maximum rate of absorption has been attained by that time, or that the speed of storage and utilization has reached an equilibrium with the rate of absorption.

Baumann, Riising and Steenbock ('34) demonstrated that a marked disappearance of vitamin A from the intestine occurs within 3 hours after its administration while the vitamin A content of the liver reaches a maximum within 6 hours in spite of the fact that large amounts of the vitamin still remain in the gastrointestinal tract for as long as 12 hours after its ingestion.

Although the absorption of vitamin A may be decreased when taken in paraffin oil (Rowntree, '31; Andersen, '39), there is only a slight loss when mineral oil and butterfat are taken separately (Jackson, '31). The presence of bile in the intestine is not as important for the absorption of vitamin A as it is for that of carotene. Schmidt and Schmidt ('30) found that vitamin A could still be absorbed by choledochocolonostomized vitamin A-deficient rats in amounts sufficient to clear

[1] Most of these data are from a thesis to be presented by A. G. Reifman to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

up the avitaminosis. Carotene, on the other hand, was ineffective in the treatment of such operated vitamin A-deficient rats (Greaves and Schmidt, '35), although when glycodesoxycholic or desoxycholic acid was added, the deficiency was overcome.

The vitamin A esters must first undergo saponification prior to absorption. According to the results of Gray, Morgareidge and Cawley ('40), an accumulation of vitamin A alcohol occurs in the gut wall at the height of absorption. In view of the fact that fat is absorbed more rapidly from the gut than vitamin A, these investigators believed that this vitamin does not play a major role in fat absorption. However, Lovern and Morton ('39) have shown that the rates of absorption of vitamin A and of neutral fat are parallel in the halibut but these results may not be applicable to higher animals since the halibut possesses no lymphatic system. The absorption of carotene but not of vitamin A is decreased in hens on a fat-free ration, according to Russell, Taylor, Walker and Polskin ('42).

The present paper is the first of a series of studies to investigate factors altering the absorption of vitamin A in the rat. The effect of concentration of the vitamin fed has been studied when concentrations varying from 100 to 1,000,000 I.U. per gram have been fed.

EXPERIMENTAL

The vitamin A solutions were fed by stomach tube to rats previously fasted for 48 hours and the amount of vitamin A remaining in the gut after 3 hours was determined. Rats from our stock colony were placed on a vitamin A-low diet [2] for 6 days prior to the fasting period in order to decrease the control level of vitamin A extractable from the gastrointestinal tract. The average control level was used as a correction factor. The procedures were similar to those employed earlier (Deuel, Hallman and Leonard, '40).

[2] Similar to the U. S. P. XI vitamin-A depletion diet except that unextracted casein was used.

Vitamin A solutions were administered in amounts of 300 mg. per 100 sq. cm. of surface area. Surface area was computed by the formula of Lee ('29) based on the weight after fasting. Absorption has been related to surface area rather than to body weight because this gives more uniform results (Deuel, Hallman and Leonard, '40). The vitamin A preparations contained approximately 100, 1,000, 10,000, 100,000 and 1,000,000 I.U. per gram. The three lowest concentrations were prepared by dilution of shark liver oil [3] of 110,000 I.U. potency with commercial hydrogenated cottonseed fat which already contained some vitamin A. For the highest levels the 110,000 I.U. shark oil and 1,004,000 I.U.[4] concentrate were fed without dilution.

After removal of the gastrointestinal tract, it was flushed with 70 cc. of freshly distilled diethyl ether. After drying with anhydrous Na_2SO_4 the extract was filtered into weighed flasks, and the Na_2SO_4 thoroughly washed with fresh ether. After removal of the diethyl ether by evaporation, the weight of the residue was determined. Except for the tests on the two highest concentrations, the residue was then saponified with alcoholic potash and extracted for 3 hours in a continuous all-glass extraction apparatus, using a low-boiling Skelly-Solve as the extracting agent. The extract was then made up to a suitable dilution with a high boiling petroleum ether fraction (60°–80° C.),[5] and the vitamin A determined with the Beckman spectrophotometer at 323 mμ.[6] Because of the high dilutions required in the tests where the 100,000 and 1,000,000 I.U. material was used, the saponification was omitted. In the last tests (series II, III and IV), the extract was made up with redistilled isopropyl alcohol so that the final dilution was a 3-to-1 mixture of alcohol and petroleum ether; in these experi-

[3] Kindly furnished by Mr. Alan Richardson of the California Packing Corporation, Emeryville, California.

[4] This was a concentrate kindly furnished by Dr. E. Geiger of the Van Camp Packing Company, Terminal Island, California.

[5] Fractionated Light Solvent, Standard Oil Company of California.

[6] This gives maximum absorption when the petroleum ether fraction is employed.

ments the spectrophotometer was read at 326 mμ which was the peak of the absorption.[7]

In order to determine whether the loss of vitamin A in the intestine might be ascribed to bacterial destruction rather than to absorption, tests were carried out in which the various preparations were introduced into the gut by stomach tube and the entire gastrointestinal tract removed as in our recovery experiments. After ligation of the esophagus, the gut was allowed to remain in an Erlenmeyer flask in an incubator at 37°C. for 3 hours, after which the contents were flushed out with ether in the usual manner. The foul odor which these preparations developed was an indication of the extensive activity of the putrefying bacteria in the intestine. Although much of the administered vitamin A remains in the stomach by this technic, the mechanical manipulation involved in the removal of the gastrointestinal tract causes considerable amounts of the administered fat to be forced into the intestine. However, in one series of tests (V), the fat was pressed out of the stomach at the start so that it would all be in the intestine during the incubation period. The various series of tests were carried on at different periods with different groups of rats. No absorption tests are recorded for series III because of the high control values for vitamin A in the intestine. Series V consists only of experiments designed to determine the extent of bacterial destruction of vitamin A. There is no difference in the significance of results of series I, II and IV but because they were carried out at different times and the sex of the animals was not the same, they are reported separately.

RESULTS

Table 1 records the various control tests, the recovery experiments, and the tests of recovery after subjecting the vitamin A preparations to the action of bacteria in the isolated gastrointestinal tracts.

[7] The authors wish to thank Fred Mattson for carrying out the determinations of vitamin A in series III, IV and V.

TABLE 1

The vitamin A and lipid recovered from the gastrointestinal contents of rats previously fasted for 2 days (A), or immediately after the administration of different concentrations of vitamin A in fat (B), or from the gastrointestinal tracts removed immediately after the administration of vitamin A but where the ligated G.I. tracts remained 3 hours in incubator at 37°C. before being flushed with ether (C).

SERIES	NO. OF TESTS	AV. BODY WT.	SEX	LIPID				VITAMIN A			
				Fed	Recovery			Fed	Recovery		
					Total	Corrected[1]	Per cent		Total	Corrected[1]	Per cent
Control tests (A)											
				mg.	*mg.*	*mg.*		*I.U.*	*I.U.*	*I.U.*	
I[2]	11	189	M	0	23.9	...	...	0	39.2	....	...
I	13	222	M	0	25.0	...	...	0	27.0	....	...
II	11	205	F	0	25.1	...	...	0	29.9	....	...
III	11	215	F	0	41.4	...	...	0	93.1	....	...
IV	18	136	M	0	18.7	...	...	0	36.7	....	...
Recovery when rats were killed immediately after fat administration (B)											
I	11	217	M	906	922	897	98.9	114	131	104	92.0
II	8	221	F	907	933	908	100.1	129	154	124	96.2
I	10	228	M	908	...	...	....	941	965	938	99.6
I	14	223	M	908	933	908	100.0	8,780	8,247	8,220	93.5
I	9	213	M	925	958	933	100.7	87,600	83,600	83,570	96.0
II	7	187	F	900	940	915	101.6	950,700	915,640	915,610	96.3
V	3	221	M	898	936	917	101.5	635	677	640	100.7
Recovery from gastrointestinal tracts after 3 hours' incubation (C)											
II	6	206	F	0	30.8	...	....	0	26.2	...	...
III	10	216	F	0	60.6	...	....	0	103	...	...
IV	7	172	M	0	34.7	...	....	0	55.8	...	...
II	16	198	F	886	946	921	103.9	123[3]	144	114	89.5
IV	5	175	M	907	915	896	98.8	185	200	164	88.7
I	6	202	M	906	927	902	99.5	114	129	102	89.5
I	4	203	M	908	918	892	98.3	941	987	960	101.9
I	2	202	M	908	932	907	99.9	8,780	8,737	8,710	99.2
I	3	226	M	927	959	934	100.7	87,600	82,730	82,730	94.4
II	2	156	F	850	873	848	99.8	904,500	869,000	888,970	96.2
V	3	253	M	898	925	890	99.2	635	679	642	101.0

[1] Corrected for amount found in gut contents of corresponding control group.

[2] Rats previously receiving stock diet were fasted 2 days. The stock diet is high in carotene and vitamin A. Other rats were kept for 6 days on carotene-free, vitamin A-low diet.

[3] Nine experiments only.

Although the average vitamin A present in the gastrointestinal tract of animals fasted 2 days after being on the stock diet amounted to 39 I.U. which was an exceptionally large correction factor for the experiments with fat containing approximately 100 I.U. of vitamin A per gram, somewhat lower values of 27 and 25 I.U. were found following a preliminary period on a low vitamin A, carotene-free diet.

However, in a later series of tests on older female rats much higher control levels of both intestinal lipid and vitamin A were found (series III). The apparent vitamin A values and lipid contents were consistently parallel. Because of the high correction values of vitamin A (average 93 I.U.) which were extremely variable, all absorption tests from this group were discarded. Similar high levels (103 I.U.) were found in the incubated gastrointestinal contents of this group of rats. These data are included to indicate one of the difficulties in obtaining satisfactory control animals. The lowest control levels in intestinal lipid were found in series IV where the animals were subjected to a preliminary enema of 5 cc. of soap solution. Tests were started 3 hours thereafter. Comparable rats were used on the control, recovery, incubation and absorption tests in each series.

The recovery of lipid approximated 100% in all tests, while the average for vitamin A varied between 92 and 100%. Because of these high values, no correction is made for the recovery in these tests as in our former studies.

The action of the intestinal bacteria on the various vitamin A preparations did not lower appreciably the vitamin A recoverable from the gastrointestinal tracts after 3 hours. Moreover, there was apparently no concurrent synthesis of new vitamin A, as the results of the control tests showed no increase in experiments on female rats and only a slight absolute increase in the tests where male rats were employed.

Table 2 gives the average rates of absorption when the various concentrations of vitamin A were fed.

There is a progressive rise in the rate of vitamin A absorption from approximately 5 I.U. per 100 sq. cm. of body

surface per hour when 100 I.U. are given to 10,000 I.U. when 950,000 I.U. are administered. The absorption of the lipid is quite uniform at 35 mg. per 100 sq. cm. per hour except for the highest level of vitamin. In this case the material, which was a saponified concentrate of fish liver oil, probably contained little readily absorbable lipid.

TABLE 2

The absorption of lipid and vitamin A over 3-hour periods by rats fed 300 mg. of either hydrogenated cottonseed oil containing different concentrations of vitamin A, or vitamin A concentrates alone.

SERIES	NO. OF TESTS	AV. BODY WT.	SEX	FAT IN MG.				VITAMIN A IN I.U.			
				Fed	Recovered[1]	Absorbed		Fed	Recovered[1]	Absorbed	
						Total	Per 100 sq. cm. per hr.			Total	Per 100 sq. cm. per hr.
II	8	194	F	883	538	346	38.5	126	67	60	6.5
IV	7	151	M	758	453	305	39.8	155	125	31	4.2
I	11	198	M	887	580	307	34.5	110	64	46	5.2
I	9	203	M	911	589	322	35.8	941	683	258	28.5
I	9	200	M	888	559	329	36.6	8,600	5,320	3,280	369
I	9	250	M	958	665	293	28.5	90,590	68,880	21,710	2,108
II	5	183	F	900	873	27	3.1	950,100	865,400	84,700	10,140

[1] Corrected for control value.

DISCUSSION

The rate of absorption of vitamin A from the gastrointestinal tract of rats is directly proportional to the concentration of the vitamin administered. Vitamin A was found to be absorbed at a speed 2,000 times as fast when 950,000 I.U. were given as when 110 I.U. were fed. At the three lowest levels the amount of vitamin A absorbed varied between 38 and 27% of the quantity fed. This value dropped to 24% for the 100,000 I.U. material and to 9% for the 1,000,000 I.U. concentrate. No statistical evaluation is necessary to establish the different rates of absorption as no overlapping occurred in any case between individual experiments of the groups where different levels of vitamin A were given.

In these tests there is no evidence of a proportionality between the absorption of vitamin A and the fat as suggested by Lovern and Morton ('39). The quantity of fat absorbed over a 3-hour period varied from 39.8 to 28.5 mg. per 100 sq. cm. while the vitamin A absorption was varying from 4.2 to 2108 I.U. per 100 sq. cm. per hour. In the tests where the highest amount of vitamin A was absorbed, there was no evidence that lipid had disappeared. However, one would expect this to be the case since most of the lipid here is actually the vitamin itself and other constituents of the unsaponifiable residue.

There is no evidence that the vitamin A which disappears from the gastrointestinal tract is the result of destruction by bacteria in the intestine. When the various vitamin preparations were introduced into the stomach by stomach tube and the intestinal tract immediately removed and allowed to stand in flasks at 37°C. for 3 hours, the same quantitative recovery was obtained as when the material was flushed out of the gastrointestinal tract immediately, although considerable putrefaction had taken place. Moreover, the putrefaction of fish livers is without any marked effect on vitamin A content [8]; it has also been demonstrated that no carotene is destroyed by intestinal bacteria over a period as long as 24 hours.[9] These data are difficult to interpret in view of the report of Hickman et al. ('42) where it was shown that the administration of α tocopherol increased the growth response to vitamin A and carotene in vitamin A-deficient rats. Similar results were also obtained by Quackenbush et al. ('42). There is no direct proof that the apparent synergistic effect may be the result of a decrease in bacterial destruction. Also, Baumann, Riising and Steenbock ('34) concluded that large amounts of vitamin A were destroyed in the gastrointestinal tract of rats because no further increase in vitamin A in the liver occurred after 6 hours although large amounts of the vitamin were still in

[8] Personal communication of Alan Richardson.

[9] Unpublished work of Dr. E. Geiger reported at the meeting of the Southern California Section of the Society for Experimental Biology and Medicine at California Institute of Technology, January 21, 1943.

the gastrointestinal tract and none was subsequently excreted in the feces. Our experiments do not preclude the possibility that the destruction of vitamin A may proceed at an accelerated rate after 3 hours. However, another explanation of the experiments of Baumann, Riising and Steenbock would seem to be that the vitamin A continued to be absorbed after 6 hours and that such destruction takes place in the liver. The body must possess some means of destroying any excess of vitamin A since no satisfactory channel for excretion is available. That such an enzyme system may be augmented in activity so that carotenoid pigments also may be destroyed has been postulated by Mattson and Deuel ('43). The destruction in this latter case must necessarily take place after absorption.

The power of the rat to absorb vitamin A is remarkable. An average of 85,000 I.U. were absorbed in a 3-hour period when the most concentrated preparations were administered. Were the rat able to store this and use it efficiently, it would represent almost a 45-year supply at a daily consumption rate of 5 I.U.

SUMMARY

The rate of absorption of vitamin A in rats was found to be proportional to the concentration of the administered material. The average absorption per 100 sq. cm. per hour was as follows: 4.2 to 6.5 I.U. for 100 unit material, 28.5 for 1,000 unit, 369 for 10,000, 2108 for 100,000, and 10140 for 1,000,000 unit.

There is no relationship between the rate of absorption of neutral fat and vitamin A. Evidence is given which indicates that vitamin A is not destroyed by intestinal bacteria over a 3-hour period.

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NUTRITIVE DIFFERENCES IN RATIONS CONTAINING UNHYDROGENATED OR HYDROGENATED FATS AS SHOWN BY REARING SUCCESSIVE GENERATIONS OF RATS

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(Received for publication October 28, 1942)

Kennedy and Palmer ('26) observed a nutritive difference between rations containing hydrogenated and unhydrogenated fat when they reported successful reproduction in three successive generations of rats on the Evans and Bishop sterility diet in which lard was replaced by a commercial hydrogenated fat. Confirmation and elucidation of these results were supplied later in reports by Evans and Burr ('27 b), Mattill ('27) and Olcott and Mattill ('34). Evans and Burr found that by increasing the lard quota from 8 to 22% in a ration consisting mainly of casein and starch the number of gestations resulting from first breedings was greatly decreased. These same authors showed that wheat germ, a known source of vitamin E, could be robbed of its effectiveness as a source of this vitamin when mixed with certain fats such as lard or oleic acid. Mattill ('27) associated this decrease in vitamin E activity with oxidative changes which accompanied the development of rancidity in unsaturated animal fats. He also reported unpublished work by Cummings in which animals were not sterile when the ordinary lard in sterility rations was replaced by hydrogenated lard. Cummings and Mattill ('31) confirmed the earlier observations of Evans and Burr ('27 a) which indicated an improvement in reproduction when hydrogenated cottonseed oil replaced cottonseed oil in the diet of

rats. A commercial hydrogenated vegetable shortening made by hydrogenating cottonseed oil to an iodine number of 70 was found to be an excellent and stable source of vitamin E (Weber, Irwin and Steenbock, '39). By comparing unhydrogenated cottonseed oil with hydrogenated cottonseed oil in rations otherwise the same, Cummings and Mattill ('31) found that hydrogenated cottonseed oil was the more reliable source of vitamin E. Olcott and Mattill ('34) reported that hydrogenation did not destroy the vitamin E in concentrates obtained from wheat germ oil and cottonseed oil. The protective factor against nutritional encephalomalacia of chicks was found to be present in hydrogenated cottonseed oil (Goettsch and Pappenheimer, '36). On a diet lacking vitamin E, resorption in the first pregnancy of mice was prevented by the administration of alpha tocopherol or by the addition of 5% hydrogenated cottonseed oil to the diet (Bryan and Mason, '40).

It has thus been demonstrated that vitamin E is present in hydrogenated cottonseed oil and in hydrogenated concentrates obtained from wheat germ oil and cottonseed oil. There are also indications that hydrogenation may result in a decreased tendency of vitamin E in the ration to be destroyed, presumably by oxidation. It appeared to the author that additional information pertaining to the relative nutritive effect caused by the presence of the unhydrogenated or hydrogenated types of fat in mixed rations could be obtained, not only by feeding the basal ration deficient in vitamin E, but also by employing rations of more adequate nutritive properties. Especially did it seem desirable to extend the study beyond the first gestation period, and, if animals were available, into succeeding generations. A study of this type would give information relating not only to fertility and reproduction, but also to the production of viable young and to lactation. For the purpose of studying the dietary properties of hydrogenated and unhydrogenated fats from the same source, it appeared that soybean oil could be chosen as an important example of an edible oil of relatively high susceptibility to oxidation.

EXPERIMENTAL PROCEDURE

In the above investigations the rations were composed of natural fats, processed fats, purified carbohydrate and protein, yeast as the carrier of vitamin B complex, and vitamins A and D in the form of cod liver oil and butterfat. These same constituents formed the basis for our present rations (table 1), but in addition a vitamin E supplement was simultaneously added in some cases as a check on the conclusion that vitamin E is destroyed in the ration containing unhydrogenated lard.

TABLE 1

Composition of rations.

	RATION A	RATION B [1]		RATION C [1]
Casein	30.0 [2]	18.0 [2]	Casein	8.0 [2]
Wheat embryo	2.0		Cottonseed meal	5.0
Dry yeast	4.0	8.0	Coconut meal	10.0
Dextrin	38.0	45.0	Yellow corn	47.0
Salt mixture	4.0 [4]	2.5 [5]	Whole milk powder	3.0
Cod liver oil	2.0	0.5	Cod liver oil	0.5
Agar		1.0	$CaCO_3$	1.0
Experimental fat	20.0	25.0	NaCl	0.5
			Experimental fat	25.0

[1] 3.0 cc. fresh milk daily; 3.0 gm. fresh liver weekly per animal.
[2] Alcohol extracted.
[3] Commercial.
[4] Steenbock no. 32 (Steenbock and Gross, '19).
[5] Hubbell, Mendel and Wakeman ('37).

In other experiments this basal ration was supplemented with fresh milk and liver in order to improve the dietary qualities of the ration.

With these rations a comparison was made between lard and hydrogenated cottonseed oil. Hydrogenated and unhydrogenated soybean oil were compared in a series of experiments where the basal ration was supplemented with fresh milk and liver and where the rations were composed largely of natural foodstuffs.

The hydrogenated soybean oil, which was a plastic product at room temperature with an iodine value of approximately 70,

came from the same lot of oil as the unhydrogenated soybean oil. This oil had previously been alkali refined and steam deodorized. The hydrogenated cottonseed oil was a commercial product having also an iodine value of approximately 70. The lard was a steam rendered product purchased on the market.

Young rats from the stock colony were placed upon the experimental diet when 25–28 days of age. The animals were selected so as to have a corresponding litter-mate animal in all groups between which dietary comparisons were made. Two male rats and three female rats composed each experimental group during the first-generation observation. The animals were fed ad libitum and allowed to run on shavings. The only data reported here relating to the reproductive history of the animals, are those given by the number of litters which were born and the number of young which were weaned. No systematic study was made either of the successful matings by the vaginal smear method or of pregnancies as shown by the erythrocyte sign. In some cases, where animals were available, the reproductive ability was observed in succeeding generations.

With the feeding of the above-mentioned experimental diets and the selection of young experimental animals which were fostered by rats receiving the stock ration, initial fertilities and birth of young were not unexpected. Vitamin E and other nutritive deficiencies in the diets could only be indicated in later gestations and succeeding generations after the original body supplies of essential nutrients were exhausted. Emerson and Evans ('39 a, b) reported that increased quantities of vitamin E were required by the female rat as the age increased.

EXPERIMENTAL RESULTS

It will be noted from the data in table 1 that ration A, composed largely of purified foodstuffs and supplemented with additional vitamin E in the form of wheat embryo, was able to maintain reproduction in the fifth generation when hydrogenated cottonseed oil composed 20% of the diet. This was

shown by the fact that each of three females from the fifth generation gave birth to a litter of normal size. When the hydrogenated cottonseed oil in ration A was replaced by an equal quantity of lard and fed to animals of the same breeding and dietary history, the number of young born from first gestations compared favorably with those animals receiving the hydrogenated fat. However, no weaning of the young occurred in any of these groups and it required the second gestation period to demonstrate differences in reproduction and weaning of the young between the lard and hydrogenated-fat groups. One-third as many young were produced from second gestations in the lard group compared to a similar period in the hydrogenated-fat group. As only one animal was weaned on the lard ration, a study of reproduction in the next generation was not possible and the feeding of the lard ration was discontinued. Similar differences were found between two groups of animals receiving lard and hydrogenated cottonseed oil, respectively, in a different kind of diet identified as ration B, which contained no vitamin E added in the form of concentrate, but did contain a supplement of milk and whole liver. Here second generation animals were obtained with each of the fat diets. However, during the observation of the second generation animals for 174 days no young were born to the females on the lard ration while the three females receiving the hydrogenated cottonseed oil diet gave birth to five litters or a total of twenty-eight young.

By using the same type of ration a similar relationship was shown between hydrogenated and unhydrogenated soybean oil in regard to dietary properties as was found in the case of unhydrogenated animal fat (lard) and hydrogenated cottonseed oil. These results are shown in table 2 under ration B. In the group of animals receiving the unhydrogenated oil only one of the three females gave birth to young. This particular female had three litters of young, none of which were weaned. During the same time, four litters or thirty-four young were born to the three females in the group

TABLE 2

Hydrogenated and unhydrogenated fats in rearing successive generations of rats.

	GENERATION	GESTATION	NUMBER OF					
			Females	Litters	Young	Young removed[1]	Litters weaned[2]	Young weaned
			Ration A[3]					
Hydrogenated	1	1	3	3	15	0	0	0
cottonseed oil	1	2	3	3	27	4	3	14
	2	1	3	2	14	2	2	11
	2	2	3	2	13	2	2	7
	3	1	3	3	21	1	2	12
	4	1	4	4	22	2	2	9
	5	1	3	3	27	7	1	6
Lard	1	1	3	3	14	0	0	0
	1	2	3	3	9	0	1	1
			Ration B[3]					
Hydrogenated	1	1	3	3	18	1	1	5
cottonseed oil	2	1	3	5	28[4]	0	2	12
Lard	1	1	3	3	16	0	3	14
	2	1	3	0[5]	0	0	0	0
Hydrogenated	1	1	3	4	34[4]	3	4	29
soybean oil	2	1	3	4	38[4]	2	4	12
	3	1	3	3	26	0	3	20
Unhydrogenated	1	1	3	3[6]	13	0	0	0
soybean oil	2[7]	1	3	3	20	1	3	16
	3	1	3	1	6	0	0	0
			Ration C[3]					
Hydrogenated	1	1	3	3	19	1	3	18
soybean oil	2	1	3	3	18	3	3	15
Unhydrogenated	1	1	3	3	23	3	3	20
soybean oil	2	1	3	3	17	2	3	15

[1] The maximum number of young allowed to be weaned per litter were six with ration A and eight with rations B and C.

[2] All or part of litter.

[3] Five hundred gram quantities were made at a time and stored in the refrigerator.

[4] Second gestation animals included.

[5] No young at 174 days of age.

[6] From one female, remaining two females had no young during 185 days.

[7] Second generation animals were taken from those which had been weaned on the hydrogenated soybean oil ration.

receiving hydrogenated soy-bean oil; twenty-nine out of the thirty young left to suckle were weaned.

Since no young were available for a second generation study in the unhydrogenated-oil group, young were taken from the hydrogenated-oil group and placed upon the unhydrogenated soybean oil ration. As a result of this procedure young were born and weaned in the group receiving the unhydrogenated oil as well as in the hydrogenated-oil group. Apparently as a result of vitamin E storage from the previous hydrogenated-oil diet and quantities of the vitamin ingested with the milk and liver, the animals were able to function for one gestation period. However, when their offspring were continued on the unhydrogenated-oil ration no animals were weaned in the following generation. Reproduction and successful weaning of the young occurred in the corresponding generation receiving hydrogenated soybean oil.

Diets made up largely of natural food products as reported in table 2 under ration C did not show any difference in nutritive properties which could be attributed to hydrogenation of the soybean oil. This was probably due to certain factors such as the presence of antioxidants in the cottonseed meal and coconut meal and the increased quantities of vitamin E and other nutrients present in the milk powder and the oil meals.

SUMMARY AND CONCLUSIONS

Rats were fed the ordinary sterility ration composed of casein, dextrin, lard, cod liver oil, salt mixture and dried yeast supplemented, in some cases, with wheat embryo and, in other experiments, with small quantities of milk and fresh liver.

In these experiments nutrition was not improved by replacing the lard with unhydrogenated soybean oil. However, when partially hydrogenated soybean oil or partially hydrogenated cottonseed oil replaced the lard, there was a decided improvement in relation to the number of young which were born, the number of young which were weaned, and the breeding of successive generations of rats.

Hydrogenation does not destroy the vitamin E present in vegetable oils. Under conditions where oxidation may cause destruction of essential factors in the diet, use of hydrogenated oil is preferable to the use of unhydrogenated vegetable or animal fats.

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THE AMINO ACIDS REQUIRED FOR GROWTH IN MICE AND THE AVAILABILITY OF THEIR OPTICAL ISOMERS [1]

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THREE FIGURES

(Received for publication January 26, 1943)

The mouse has been used much less than the rat in nutritional investigations, but has proved especially valuable in certain types of cancer studies, a few of which have involved the factor of diet. It would seem desirable, therefore, to know whether the need of the growing mouse for protein can be met by mixtures of purified amino acids suitable for the growing rat (Rose, '38).

The expediency of feeding some of the amino acids in the racemic form justifies determining also whether the mouse can utilize both optical antipodes of an essential amino acid, or only the natural isomer. Although such information is relatively complete for the rat (Rose, '38), few tests of this sort have been made in the mouse. It has been found that *d*(+)-tryptophane and *d*(+)-histidine allow less rapid growth than the natural *l*(—) forms, and that unnatural *d*(—)-lysine fails to support growth (Totter and Berg, '39). At the outset an additional incentive for making further comparisons in the mouse was presented by reports, since challenged, that tumors

[1] The experimental data in this paper are taken from a dissertation submitted by Clifford D. Bauer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

An abstract has been published (Bauer and Berg, '42).

which arise spontaneously in certain strains of this species contain unnatural amino acids.

The experiments recorded in this communication were directed toward achieving these objectives. They show that a number of amino acids are indispensable to the mouse and they afford qualitative comparisons of the availability for growth of the optical antipodes of methionine, phenylalanine, valine, leucine, isoleucine, and threonine.

EXPERIMENTAL

The mixture of purified amino acids used was patterned after that employed by Rose and Rice ('39) in growth studies on the rat, the chief difference being that the percentage of each amino acid was increased to provide the reputedly greater protein need of the mouse. All of the amino acids were prepared in the laboratory, except the *dl*-alanine and the *l*(+)-glutamic acid which were commercial products.[2] Nine of the other amino acids were synthesized and nine were isolated from natural sources. Most of the isolation procedures had been used repeatedly in the laboratory, and tests made at various times had shown that if they were applied with reasonable care they were capable of yielding products free of admixed amino acids and other contaminants. The aspartic acid was isolated from the anodic liquors obtained as a by-product in the preparation of arginine, histidine, and lysine. Synthesis of the components of the leucine fraction was chosen to avoid the risk of incomplete fractionation and contamination with methionine encountered in their isolation from natural sources. In several instances synthesis appeared to be the only suitable method of preparation. All of the amino acids were beautiful white products and all had been recrystallized at least once or twice oftener than necessary to show proper melting point, acceptable rotation, and theoretical content of amino or total nitrogen. Composition of the basal amino acid mixture and references to methods of isolation or synthesis, several of which were modified in

[2] Pfanstiehl.

detail, are recorded in table 1. The optical antipodes were obtained by formylating the racemic mixtures and resolving them with brucine. References to specific procedures and data pertaining to optical rotations are presented in table 2.

The basal diet consisted of the amino acid mixture, 33.65; sucrose, 15; starch, 13.35; cod liver oil, 5.0; hydrogenated

TABLE 1

Composition of the amino acid mixture.[1]

	NATURAL AMINO ACID FRACTION	AS FED
	gm.	*gm.*
Glycine (Orten and Hill, '41)	0.15	0.15
dl-Alanine	0.30	0.60
dl-Serine (Schiltz and Carter, '36)	0.15	0.30
dl-Valine (Marvel, '40)	1.50	3.00
dl-Leucine (Marvel, '41 b)	1.95	3.90
dl-Isoleucine (Marvel, '41 a)	1.35	2.70
dl-Norleucine (Marvel and du Vigneaud, '25)	0.60	1.20
l(—)-Cystine (Okabe, '27–'28)	0.70	0.70
dl-Methionine (Barger and Weichselbaum, '34)	0.90	1.80 [1]
l(—)-Proline (Town, '28)	0.30	0.30
l(—)-Hydroxyproline (Klabunde, '31)	0.15	0.15
l(—)-Aspartic acid (Jones and Moeller, '28)	0.30	0.30
l(+)-Glutamic acid	3.00	3.00
dl-Phenylalanine (Marvel, '41 c)	2.25	4.50 [1]
l(—)-Tyrosine (Cox and King, '30)	1.50	1.50
l(+) Arginine, fed as the monohydrochloride (Cox, King and Berg, '29)	0.75	0.90
l(—)-Histidine, fed as the monohydrochloride monohydrate (Cox, King and Berg, '29)	0.75	1.05
l(+)-Lysine, fed as the monohydrochloride (Cox, King and Berg, '29)	2.25	2.80
l(—)-Tryptophane (Cox and King, '30)	0.60	0.60
dl-Threonine (Carter and West, '40)	1.05	2.10
Sodium bicarbonate		2.10
	20.50	33.65

[1] Subsequent changes made in the content of *dl*-methionine and *dl*-phenylalanine in some of the studies are indicated in the text.

TABLE 2

Specific rotation of the isomers used in the feeding tests.

AMINO ACID	$[\alpha]_D$ Found	$[\alpha]_D$ Recorded in reference	CONCENTRATION PER 100 CC. OF SOLUTION As read	CONCENTRATION PER 100 CC. OF SOLUTION Recorded in reference	SOLVENT USED [1]	TEMP. OF SOLUTION READ [1]	REFERENCE
	degrees	*degrees*	*gm.*	*gm.*		°C.	
l(−)-Methionine	− 7.7	− 7.5	0.8	0.8	H_2O	25	Windus and Marvel, '31
d(+)-Methionine	+ 8.12	+ 8.12	0.8	0.8	H_2O	25	Windus and Marvel, '31
l(−)-Phenylalanine	−34.7	−35.14	1.6	1.9	H_2O	20	Fischer and Schoeller, '07
d(+)-Phenylalanine	+33.8	+33.5	2.0	Not recorded	H_2O	26	du Vigneaud and Meyer, '32
l(−)-Leucine	+15.3	+15.8	4.48	4.54	20% HCl soln	20	Fischer and Warburg, '05
d(+)-Leucine	−15.2	−15.5	4.00	4.39	20% HCl soln	20	Fischer and Warburg, '05
l(+)-Valine	+28.0	+28.7	3.20	3.56	20% HCl soln	20	Fischer, '06
d(−)-Valine	−28.22	−28.4	2.80	3.38	20% HCl soln	20	Fischer, '06
l(+)-Isoleucine	+40.03	+40.61	1.60	5.09	20% HCl soln	20	Locquin, '07
d(−)-Isoleucine	−38.3	−40.86	1.60	4.53	20% HCl soln	20	Locquin, '07
d(−)-Threonine [2]	−28.0	−28.3	2.00	Not recorded	H_2O	26	West and Carter, '37
l(+)-Threonine	+28.2	+28.4	2.00	Not recorded	H_2O	26	West and Carter, '37

[1] The solvents and temperatures used in the determinations were those recorded in the reference indicated.

[2] *d*(−)-Threonine is the natural modification. In all other instances, the *l* form is the natural isomer.

cottonseed oil,[3] 26.0; salt mixture (Hubbell, Mendel and Wakeman, '37), 5.0; and agar, 2.0%. The vitamin B complex was supplied in the form of two pills daily, each containing 25 mg. of a rice polish concentrate,[4] 0.1 mg. of nicotinic acid, 0.02 mg. of thiamine chloride, 0.02 mg. of riboflavin,[5] corn syrup (50 mg.), and enough starch (usually about 100 mg.) and water to form pellets of suitable size and consistency for molding. The corn syrup lessened the tendency to crumble which obtained when starch alone was added. In a few of the earlier studies gum acacia was used for a similar purpose.

The albino mice were of the Bagg strain.[6] The experimental and control animals were litter mates; usually three or four litters were represented in each test. Individual weights at the start of the experimental period ranged from 8 to 14 gm. Each study was continued for 40 days; weight changes and food consumption were recorded at 4-day intervals. The temperature of the room was thermostatically controlled and care was taken to avoid drafts.

When an optical isomer of any amino acid was tested for its capacity to promote growth, the *dl* form was omitted from the amino acid mixture and the active enantiomorph was incorporated at the natural amino acid level indicated in table 1. Control animals were fed a mixture of amino acids devoid of all forms of the one tested. The starch content of the diet was varied as required to compensate for any change in amino acid content.

The growth data are shown in figures 1 and 2. At the levels fed, *d*(+)- and *l*(−)-methionine were apparently equally effective. This was true also of the antipodes of phenylalanine. In the phenylalanine series the control animals which received neither phenylalanine nor tyrosine lost weight more rapidly than the controls which received tyrosine,

[3] Crisco.

[4] Ryzamin-B, from Burroughs-Wellcome Co., Tuckahoe, New York.

[5] A portion of the riboflavin was from a lot made available to the laboratory by Merck and Company.

[6] It is a pleasure to acknowledge our indebtedness to Dr. Halsey J. Bagg of the New York Memorial Hospital who kindly supplied us the foundation stock.

but not phenylalanine. When considered with subsequent tests which show that growth can occur on diets devoid of tyrosine, this observation suggests that the lack of tyrosine imposes a greater need for phenylalanine. Since both isomers of methionine and phenylalanine were effective in promoting growth, only half as much of their *dl* forms was fed in the later studies on isoleucine and threonine.

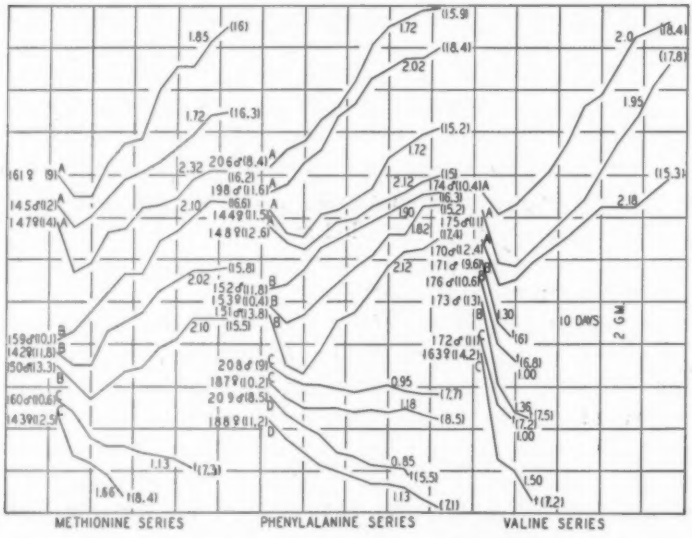

Fig. 1 Comparative rates of growth in mice fed mixtures of purified amino acids containing the natural (A), unnatural (B), or neither (C) antipode of methionine, phenylalanine, or valine. D represents the omission of both phenylalanine and tyrosine. The initial and final weights of the mice are enclosed in parentheses. The daggers indicate death. The unenclosed number which appears near the end of the curve or follows the final weight indicates average daily food consumption in grams.

The tests of the isomers of valine (fig. 1), and of leucine, isoleucine, and threonine (fig. 2) show clearly that only the natural forms of these acids can support growth. In all cases, mice fed the unnatural modifications lost weight as rapidly as control animals fed diets lacking both isomers. Marked

differences in degree of nutritive failure were apparent. When valine or leucine was omitted, or only the unnatural isomer of either was fed, the decline in weight was rapid; marked loss of appetite, extreme weakness, and lack of coordination were noted; and in nearly every instance death occurred in a few days. Lack of natural threonine induced

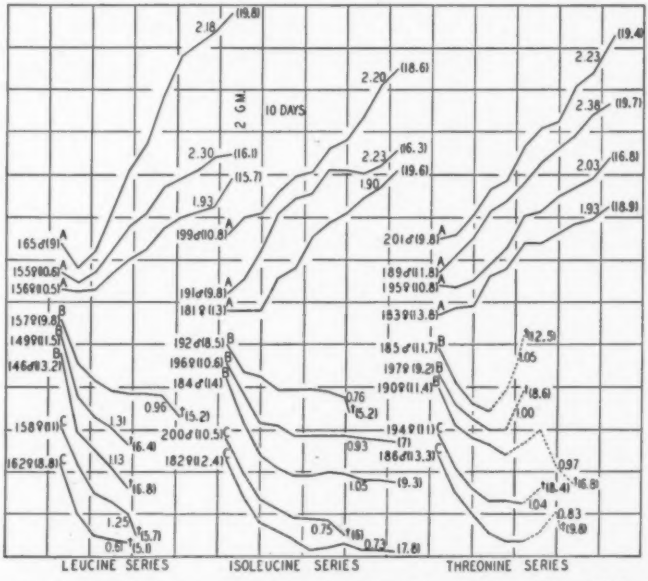

Fig. 2 Comparative rates of growth in mice fed mixtures of purified amino acids containing the natural (A), unnatural (B), or neither (C) antipode of leucine, isoleucine, or threonine. The initial and final weights of the mice are given in parentheses. The daggers indicate death. The broken lines in the threonine series of curves indicate weight changes associated with the development or re-solution of ascites and edema. The average daily food consumption in grams is indicated by the number near the end of the curve.

a rapid weight loss for 12 to 20 days, followed by a gain. In the latter interval the animals acquired a general puffy appearance; abdominal distension was particularly marked. Three of the five mice died suddenly while still gaining weight; the other two lost weight again before death. Post mortem

examination showed pronounced edema and ascites in the first three animals. Edema is known to occur in malnutrition (Maver, '20) and has been developed experimentally in rats maintained on low-protein diets (Frisch, Mendel and Peters, '29). Palpable edema had not previously been noted by us, either in mice or in rats fed diets deficient in an essential amino acid; a manifestation so striking as was obtained in this instance could hardly have been overlooked.

The relatively large weight losses of many of the mice during the first 4–8 days on the synthetic diets may be attributed to their failure to accept the diet readily. Mice frequently reject synthetic diets which are potentially able to support growth and which are eaten readily by rats; they seem slower to adapt themselves to dietary changes. Good correlation between growth rate and food consumption was not obtained. This may be attributable in part to differences in growth proclivities; also in part to errors inherent in weighing back scattered food.

Figures 1 and 2 show clearly that methionine, phenylalanine, valine, leucine, isoleucine, and threonine are required by the mouse for growth. Previous studies have indicated the need for tryptophane (Willcock and Hopkins, '06–'07; Wheeler, '13) and histidine (Geiling, '17), and may be interpreted also as suggesting that lysine is necessary. Figure 3 shows that the mouse possesses considerable capacity to synthesize arginine; on mixtures of purified amino acids free of arginine, growth was as rapid as on mixtures containing it, but was not maximal. On a diet which contained 20.2% of commercial casein, 0.3% of cystine, and 8% of yeast three mice of the same strain, weighing 10.6, 11.2 and 11.4 gm. at the outset, gained 14.8, 19.2 and 20.8 gm., respectively, in 40 days, or about twice as much as those fed the amino acid mixture. Whether the capacity of the mouse to synthesize arginine is great enough to allow such rapid growth on an arginine-free diet can be determined only by further study. The cause of the greater growth on the casein-cystine-yeast diet is not of immediate concern, but two possibilities may be mentioned:

(1) The commercial casein contained at least a tenth more total nitrogen than was present in the natural amino acids of the purified amino acid mixture; and the dried yeast contributed additional protein of different composition. Hence, the natural amino acids supplied by the casein-cystine-yeast diet differed in quantity and in distribution from those furnished in the purified amino acid mixture. (2) Casein and yeast also contain considerable phosphorus. Recently Jones and Foster ('42) have presented convincing evidence that the Hubbell, Mendel and Wakeman ('37) salt mixture supplies too little phosphorus for optimum growth in rats, even though fed at levels as high as 4% in diets otherwise low in phosphorus; on the other hand, levels of 2% supported excellent growth when the diet contained other sources of phosphorus, such as casein and yeast. The need of the mouse for minerals is generally considered to be more intensive than for the rat. It is therefore possible that 5% of the salt mixture limited the growth of the mice on the purified amino acid diet which contained no other sources of phosphorus, but was entirely adequate for the mice fed the phosphorus-rich casein and yeast.

In a final test on purified amino acids, mice were fed a mixture of the nine amino acids known to be essential for growth, plus arginine, as the sole source of protein nitrogen. The *dl*-methionine and the *dl*-phenylalanine were fed at levels of 1.8 and 3.4% of the diet, respectively, and the other eight amino acids at levels 50% higher than those indicated in the "as fed" column of table 1. The free amino acids represented 30.3% of the diet and the isomers known to be effective (including both forms of methionine and phenylalanine), 15.6%. The only other dietary changes made were compensatory adjustments in the sodium bicarbonate and starch contents. The curves in figure 3 show that the mice grew slowly on the diet.

In the previous studies of Rose ('38) rats fed these same ten amino acids at an active level of but 11.2% of the diet "gained in weight just as rapidly as when all of the protein components were supplied preformed." The metabolic origins

of the dispensable amino acids which were omitted from the diet are not clearly defined. Growing rats fed diets deficient in cystine produce cystine for tissue synthesis if methionine is available (Beach and White, '39). Apparently tyrosine can be produced metabolically from phenylalanine (Moss and Schoenheimer, '40) and glutamic acid from histidine (Edlbacher and Neber, '34). Perhaps our mice grew at a diminished rate because one or more of the essential amino acids was

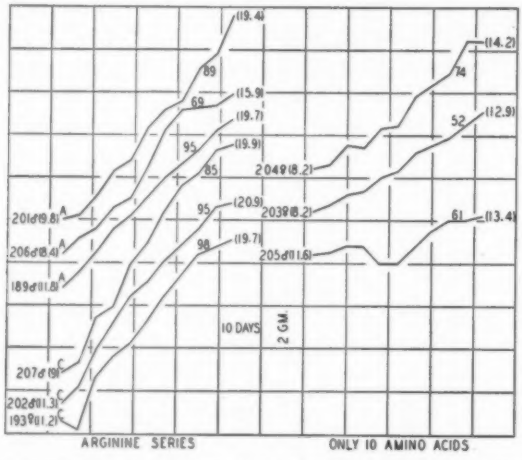

Fig. 3 Comparative rates of growth of mice fed a mixture of twenty purified amino acids (A), a similar mixture lacking arginine (C), or a mixture of only the ten amino acids essential for the rat, including arginine. The initial and final weights are given in parentheses. The total weight of food consumed in grams during the 40-day period is indicated near the end of each curve.

not supplied in an amount sufficiently greater than normal to fully offset its diversion for synthesis of certain dispensable amino acids omitted from the diet. The slower growth could, however, have been caused by some other factor, such as slow rate of synthesis or expenditure of excessive energy in synthesizing so many amino acids simultaneously. In any event, the fact that growth occurred at all suggests that the amino acids which were not fed pre-formed were synthesizable to an appreciable degree.

SUMMARY

Moderate growth was obtained in mice fed mixtures of twenty purified amino acids as the source of protein nitrogen. Both optical forms of methionine and phenylalanine, but only the natural forms of valine, leucine, isoleucine, and threonine, could be utilized for this purpose. The rate of growth was not retarded by the removal of arginine. Evidently, therefore, the mouse is able to synthesize arginine at a fairly rapid rate.

Slow growth occurred when the source of protein nitrogen was limited to the seven amino acids named above and lysine, tryptophane, and histidine. Hence, none of the ten amino acids omitted from the latter mixture could have been absolutely indispensable.

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BIOTIN CONTENT OF MEAT AND MEAT PRODUCTS [1]

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(Received for publication January 27, 1943)

The biotin content of a few meat products has been determined by Cheldelin and Williams ('42) and Lampen, Bahler and Peterson ('42). Since the importance of biotin in nutrition has continued to receive recognition it seemed desirable to have additional information on the distribution of this vitamin in meat and meat products and also to study the retention of biotin during cooking. Several meats were assayed for their biotin content, and the retention of this vitamin was determined during roasting, braising and broiling of pork hams and loins.

EXPERIMENTAL

The Lactobacillus casei method described by Shull, Hutchings and Peterson ('42) and modified by Shull, Miller and Peterson ('43) was used for the biotin determinations.

The biotin was liberated from the meats by treating with 6N H_2SO_4 and autoclaving for 2 hours at 15 pounds pressure. Digestion with pepsin, clarase or pancreatin did not liberate all of the biotin. A representative sample of meat was treated with 2N, 4N, and 6N H_2SO_4 and autoclaved for 2, 4, and 6 hours to determine the best method of hydrolysis in order to give the maximum liberation of biotin. Autoclaving with

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by a grant from the National Livestock and Meat Board, made through the National Research Council.

4 or 6 normal H_2SO_4 for 2 hours gave maximum liberation, while longer periods of autoclaving resulted in considerable destruction of the biotin. The procedure adopted for liberation of biotin was a 2-hour autoclaving of the sample in 6N H_2SO_4. This procedure was used for all samples analyzed. Satisfactory recoveries of added biotin were obtained when this procedure was used.

After treating the samples with 6N H_2SO_4, the digestion mixture was neutralized with NaOH. It was necessary to determine whether the Na_2SO_4 formed would act as a stimulant or an inhibitor of Lactobacillus casei. The maximum amount of Na_2SO_4 present in any assay tube was 0.7% and it was found that Na_2SO_4 concentrations ranging from 0.2–1.8% had no effect on the assay.

Meat samples 45–257 have been previously analyzed for their vitamin content (Waisman and Elvehjem, '41; McIntire, Schweigert, Henderson and Elvehjem, '43; and Schweigert, McIntire and Elvehjem, '43). Samples 45–168, 258 and 259 were prepared for analysis by removing all visible fat and connective tissue from the outer surface of the meats. These samples were analyzed dry and the millimicrograms of biotin per gram of fresh meat were calculated from the biotin content per gram of dry meat. Samples 170–257 were prepared for analysis by removing the rind and bones from the pork hams and loins. About ½ inch of fat was left on the outer surface. The samples were then passed three times through an electric meat grinder and thoroughly mixed. These samples were analyzed as prepared and the biotin content per gram of dry meat was calculated from the biotin content of the moist meat. The results of the analysis of the meats are found in table 1.

The selection and preparation of the meats used in the cooking experiment have been recently reported (McIntire et al., '43). The loin and hams were cooked without seasoning by standard cooking procedures (Methods of Cooking and Testing Meat for Palatability, '33); the braising procedure has been described by McIntire et al. ('43). In all cases the

TABLE 1

Biotin content of meat samples.

DESCRIPTION OF SAMPLE	SAMPLE NO.	MμG./GM. FRESH	MμG./GM. DRY
Kidney			
Pork	62	1348	6235
Pork	137	1250	6225
Beef	81	923	4050
Liver			
Lamb	96	1270	4375
Beef	131	966	3315
Beef	150	1180	4280
Beef	152	880	2980
Fried beef	121	1370	3195
Veal	158	752	2820
Pork	122	946	2995
Pork	153	790	2550
Pork	159	805	2980
Heart			
Pork	104	182	815
Veal	155	150	715
Beef	133	78	405
Beef	143	68	358
Veal			
Round	103	77	287
Round	134	63	288
Round	259	38	145
Fried chops	45	190	246
Beef			
Round	128	34	144
Round	190	63	251
Round	258	29	106
Rib	189	34	124
Pork ham			
Fresh	175	69	158
Fresh	198	44	90
Fresh	185	44	86
Fresh [1]	236	45	121
Broiled	200	65	112
Broiled [1]	239	60	111
Broiled	177	85	175
Broiled	187	75	143
Pan broiled	178	74	139
Pan broiled	201	75	126
Braised	188	68	126
Pork loin			
Fresh	170	56	144
Fresh	180	61	119
Fresh	193	47	95
Fresh [1]	230	43	96
Braised	183	67	110
Braised	196	54	85
Braised [1]	233	49	83
Broiled	172	81	162
Broiled	182	74	124
Broiled	195	61	101
Broiled [1]	235	58	105
Roast	171	55	115
Roast	181	69	134
Roast	194	58	114
Roast [1]	231	45	94
Pan broiled	173	83	153
Cured hams			
Smoked	102	96	246
Cured [1]	251	46	89
Cured [1]	252	61	136
Roast cured [1]	250	59	106
Roast cured [1]	253	83	151
Fried cured [1]	256	100	176
Fried cured [1]	257	85	155
Lamb			
Breast [1]	243	21	146
Leg	80	59	224
Miscellaneous			
Beef pancreas	113	137	596
Dark chicken meat	66	101	393
Light chicken meat	68	113	459
Beef spleen	76		245
Beef lung	138	59	290
Beef brain	144	61	288
Beef tongue	82	33	119
Fillet of cod	135	49	287
Salmon steak	136	59	211

[1] Analyzed moist.

drippings were retained for analysis. The moisture and fat determinations and preparation of the meats and drippings for analysis are the same as reported by McIntire et al. ('43), and Schweigert et al. ('43). The detailed results of one of the cooking series are given in table 2.

TABLE 2

Biotin content of meat before and after cooking.

METHOD OF COOKING	BEFORE COOKING			AFTER COOKING					
	Weight of fresh meat	Biotin in meat	Biotin: total in meat	Weight of cooked meat	Biotin of cooked meat	Biotin total in cooked meat	Total biotin in drippings	Retained in cooking	Total retained
	gm.	mμg./gm.	μg.	gm.	mμg./gm.	μg.	μg.	%	%
Fresh loin, roasted	1105	43	47.6	859	45	38.7	0.61	81	83
Fresh loin, braised	332	43	14.3	242	49	11.9	0.70	83	88
Fresh loin, broiled	347	48	16.7	176	58	10.2	0.32	61	63
Fresh ham, roasted	2348	45	106.0	1542	48	74.2	3.15	70	73
Fresh ham, broiled	801	44	35.2	503	60	30.2	0.55	86	87

TABLE 3

Biotin retention during cooking.

	DETERMINATION	BIOTIN RETAINED IN MEAT ALONE	TOTAL RETAINED
		%	%
Roast loin	1	80	82
	2	81	83
Braised loin	1	76	77
	2	83	88
Broiled loin	2	61	63
Roast ham	1	75	76
	2	70	73
Braised ham	1	76	78
Broiled ham	2	86	87

The biotin retained in the meat alone after cooking was calculated by dividing the total micrograms of biotin in the entire piece of cooked meat by the total micrograms in the uncooked meat. The total per cent retained in the meat plus drippings was calculated by adding the micrograms of biotin in the drippings and fresh cooked meat and dividing the sum by the total micrograms in the uncooked meat. The per cent retained in the meat alone and the per cent retained in the meat plus drippings in two cooking series are found in table 3.

DISCUSSION

The biotin content of meat products agrees fairly well with the results reported by Lampen et al. ('42). In most cases our results were higher, particularly in the case of pork kidney, beef kidney and heart. Our results agree very well with the values reported by Cheldelin and Williams ('42), who used Saccharomyces cerevisiae as the test organism. The only discrepancy exists in the biotin content of light chicken meat. They found 54 mμg. of biotin per gram of fresh tissue as compared to 113 mμg. per gram which we obtained. The results obtained with Saccharomyces cerevisiae and Lactobacillus casei methods are, therefore, in very good agreement.

Liver and kidney were found to be the best sources of biotin. Pork kidney averaged 6230 mμg. per gram of dry tissue as compared to 4050 mμg. of biotin per gram for beef kidney. Lamb liver contained 4375 mμg. per gram, beef liver averaged 3530, veal liver 2820, and pork liver averaged 2840 mμg. per gram of dry tissue.

Heart, pancreas, and dark and light chicken meat are good sources of biotin. Beef spleen, lung, brain, and tongue have about the same amount of biotin in millimicrograms per gram of fresh tissue as lamb, beef, veal and pork muscle meats. Pork hams and loins averaged 50 mμg. per gram of fresh tissue.

From 61 to 86% of the biotin was retained in the meat alone after cooking, the lowest retention being observed after broil-

ing pork loin. The total retention of biotin in the meat plus drippings ranges from 63 to 88% of the biotin present in the uncooked meat. The amount of biotin found in the drippings was very small, ranging from 1 to 5% of the total amount of biotin present in the original meat. This indicates that a very small amount of biotin leached out of the meat during cooking in contrast to higher amounts of thiamine, nicotinic acid and riboflavin that were found in the drippings as reported by McIntire et al. ('43) and Schweigert et al. ('43).

The destruction of biotin during cooking is very likely due to the formation of the oxidized form of biotin, which is not utilized by Lactobacillus casei (Nielsen, Shull and Peterson, '42).

SUMMARY

1. Biotin content of various meat products has been determined by the Lactobacillus casei method.

2. Kidney and liver were the richest sources of biotin. Heart, pancreas, and dark and light chicken meat were good sources. Beef spleen, lung, brain, and tongue contain about the same amount of biotin as pork, beef, veal and lamb muscle meats. Pork loins and hams averaged 50 mμg. of biotin per gram of fresh tissue.

3. An average of 77% of the biotin was retained in the meat alone after cooking, and an average of 80% was retained in the meat plus drippings after cooking.

4. From 1–5% of the biotin present in the original meat was recovered in the drippings after cooking.

ACKNOWLEDGMENT

The authors wish to express their thanks to Dr. Catherine J. Personius, Professor of Home Economics, for cooking the meats, and to Mr. Robert W. Bray, of the Animal Husbandry Department, for selecting and cutting the meats used in the cooking experiments.

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THE RETENTION OF VITAMINS IN MEATS DURING STORAGE, CURING AND COOKING[1]

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(Received for publication February 8, 1943)

The retention of vitamins during cooking of fresh pork hams and loins has recently been reported by McIntire, Schweigert, Henderson and Elvehjem ('43). Very little information is available on the retention of vitamins during storage and curing, or cooking of cured meats. Dann and Handler ('42) in preliminary experiments have indicated a definite loss of nicotinic acid on refrigerator storage of meats for 1 week and suggest possible destruction of nicotinic acid on commercial aging of meat. We decided to make a study of thiamine, nicotinic acid and riboflavin retention in pork hams during storage, curing and cooking of the cured hams.

EXPERIMENTAL

Pork hams were taken from carcasses weighing approximately 190 pounds. Paired hams were used throughout the experiment. Two fresh hams were analyzed immediately and the two corresponding hams were stored for 14 days in a freezer (— 4°C.) in order to determine the vitamin retention during storage.

Two different hams were stored for 14 days and the corresponding two hams were cured commercially in order to study vitamin retention during the curing process. Four

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Livestock and Meat Board made through the National Research Council.

additional hams were cured. Two of these were analyzed uncooked and the two corresponding hams were used for cooking tests. In all cases the right and left hams were alternated in the duplicate tests.

The hams were cured commercially by placing them in a pickling solution for 12 days. They were then removed, washed with warm water, and placed in a smoke house for 18 hours. At that time the internal temperature of the ham reached 145°F. They were left at that temperature for 2–4 hours, then removed and cooled.

Roasting of the cured hams was carried out under standard conditions without seasoning (Meat and Meat Cookery, '42). Two of the cured hams were roasted in an electric oven at 275°F. until the internal temperature of the meat reached 170°F. The time required for roasting was 7 hours and 50 minutes for one ham and 7 hours and 20 minutes for the other. The drippings from both roasts were retained for analysis.

Two slices ½ inch thick from another cured ham were removed parallel to the aitch bone for frying (panbroiling). Similar cuts from the corresponding fresh stored ham were removed for analysis to study over-all vitamin retention from fresh stored to cured fried samples. The frying was carried out as follows: The pan was preheated on an electric stove for 2 minutes and the cured slices were browned on each side at moderate heat for 3 minutes. The rest of the frying was carried out at low heat until the meat appeared well-done. The total cooking time for the ham slices was 16 minutes. The drippings from frying were retained for vitamin analysis.

All hams were prepared for analysis by removing the rind and bones and passing the entire ham three times through an electric meat grinder. The preparation of the drippings for analyses and the moisture and fat determinations were carried out as previously reported (McIntire et al., '43). Vitamin, fat and moisture analyses were run immediately on all samples. The residual solids were calculated by subtracting the per cent of fat and water from 100.

The thiamine contents of the samples and drippings were determined by the method of Hennessy ('42) with modifications of McIntire et al. ('43), the nicotinic acid by the method of Snell and Wright ('41), and the riboflavin method used was that of Snell and Strong ('39) with modifications reported by McIntire et al. ('43).

TABLE 1

Detailed analysis of meat.

DESCRIPTION OF SAMPLE	SAMPLE NO.	PROXIMATE ANALYSIS			THIAMINE		NICOTINIC ACID		RIBOFLAVIN	
		water	fat	residual solids	μg. per gm. fresh	μg. per gm. dry [1]	μg. per gm. fresh	μg. per gm. dry [1]	μg. per gm. fresh	μg. per gm. dry [1]
		%	%	%						
Hog B, fresh	247	49.8	34.0	16.2	6.7	41.3	41	254	2.5	15.4
Hog B, stored	246	52.1	32.1	15.8	5.9	37.3	36	227	2.2	13.9
Hog F, fresh	254	55.1	27.8	17.1	9.5	55.5	42	246	2.6	15.2
Hog F, stored	255	54.8	28.5	16.7	9.0	53.8	40	240	2.1	12.6
Hog C, stored	248	55.8	29.1	15.1	7.8	51.6	36	238	2.0	13.2
Hog C, cured	249	53.5	27.6	18.9	7.2	38.0	38	201	2.3	12.2
Hog D, cured	251	48.2	35.3	16.5	4.7	38.9	21	127	2.0	12.1
Hog D, cured, roast	250	44.7	34.7	20.6	4.7	26.4	26	126	2.0	9.7
Hog E, cured	252	55.2	25.9	18.9	7.2	39.5	34	180	1.9	10.1
Hog E, cured, roast	253	45.0	33.1	21.9	5.0	19.7	36	164	2.1	9.6
Hog C, fresh steak	244	52.5	31.2	16.3	8.5	52.0	37	227	2.1	12.9
Hog C, cured fried steak	256	43.4	24.7	31.9	9.9	31.0	52	163	2.9	9.1
Hog C, fresh steak	245	53.9	29.6	16.5	9.4	57.2	40	242	2.1	12.7
Hog C, cured fried steak	257	45.3	25.0	29.7	9.9	33.3	52	175	2.8	9.4

[1] Calculated on the basis of residual solids.

All of the vitamin analyses were made directly on samples without drying and the vitamin content per gram of dried, ether extracted meat was calculated from these figures. Detailed results are given in table 1. The vitamin content of the meats before and after cooking is summarized in table 2. The per cent of vitamin retention in the meat after storage was

calculated from the vitamin analysis per gram of fresh meat. The per cent retention in the meat after curing was calculated from the vitamin content of the residual solids, although the actual analyses were made on the wet basis.

TABLE 2

Vitamin content of meats before and after cooking.

	CURED HAM ROASTED	CURED HAM ROASTED	CURED HAM STEAK FRIED	CURED HAM STEAK FRIED
		Before cooking		
Sample no.	251	252	249	249
Weight (gm.)	8288	5489	412	479
Thiamine in meat (total mg.)	38.9	39.5	2.97	3.45
Nicotinic acid in meat (total mg.)	174	187	15.6	18.2
Riboflavin in meat (total mg.)	16.5	10.4	.95	1.10
		After cooking		
Sample no.	250	253	256	257
Weight (gm.)	5568	3930	248	308
Thiamine in meat (total mg.)	26.1	19.7	2.46	3.05
Thiamine in drippings (total mg.)	5.1	4.3	.11	.25
Nicotinic acid in meat (total mg.)	145	141	12.9	15.7
Nicotinic acid in drippings (total mg.)	31.9	28.0	1.68	1.89
Riboflavin in meat (total mg.)	11.1	8.3	.72	.86
Riboflavin in drippings (total mg.)	1.48	1.15	.09	.10

The per cent retained in the meat alone after cooking was calculated by dividing the total milligrams of the vitamin in the entire piece of cooked meat by the total milligrams in the uncooked meat. The total per cent retained was calculated by adding the milligrams of vitamin in the drippings and fresh cooked meat and dividing the sum by the total milligrams in the uncooked meat.

The over-all retention from storage to cured fried samples was calculated from the vitamin content of the residual solids. The per cent retention after storage, curing and cooking and over-all retention from stored to cured fried samples are shown in table 3.

TABLE 3

Vitamin retention in per cent.

PROCESS	DETERMINATION	THIAMINE		NICOTINIC ACID		RIBOFLAVIN	
		Retained in meat	Retained in meat plus drippings	Retained in meat	Retained in meat plus drippings	Retained in meat	Retained in meat plus drippings
Storage	1	88		88		88	
	2	95		95		81	
Curing	1	73		84		92	
Over-all retention Fresh stored to cured fried	1	60		72		74	
	2	58		72		74	
Roasting	1	67	80	83	102	67	76
	2	50	61	75	90	80	91
Frying	1	83	87	83	94	76	85
	2	88	96	86	97	78	87

DISCUSSION

The values found for riboflavin and nicotinic acid in fresh hams are about the same as those previously reported (McIntire et al., '43). However, the thiamine content of 6.7 μg. per gram for one of the fresh hams is lower than that for any fresh hams previously analyzed. There is very little, if any, change in water and fat during storage. The residual solids on the cured samples are higher than those on the fresh, indicating loss in water and fat during curing. Both of the fried samples lost considerable water and some fat.

Sample numbers 244, 245, and 248 are all from the same fresh stored ham. The center slices (samples 244 and 245) are higher in thiamine content than the rest of the ham, 8.5 and 9.4 μg. per gram as compared to 7.8 for the remainder of the ham. This would indicate considerable variation in

the vitamin content of different sections of the ham. The nicotinic acid values are more constant, 37 and 40 μg. per gram of ham slice, and 36 μg. per gram for the remainder of the fresh ham. Riboflavin values were still more consistent.

The thiamine, nicotinic acid and riboflavin contents of the fresh and fresh stored hams were similar, although the stored meats showed slightly lower values in all cases.

In the curing process one of the hams was lost; consequently, only one pair of hams could be used for studying retention in curing. Seventy-three per cent of the thiamine, 84% of the nicotinic acid, and 92% of the riboflavin were retained during curing. The values are nearly the same as were observed during cooking, the curing process being actually an extended, low temperature cooking.

Fifty and 67% of the thiamine in the cured ham were retained in the meat alone after roasting, while 83 and 88% of the thiamine were retained in the fried ham steaks. An average of 82% of the nicotinic acid and 75% of the riboflavin was retained in the meat alone after roasting and frying. From 61 to 80% of the thiamine was retained in the meat plus drippings after roasting, and an average of 92% after frying, while 96% of the nicotinic acid and 85% of the riboflavin were retained after roasting and frying. Approximately the same time was required to roast each of the hams, and this may account for the lower retention of thiamine in the smaller ham. The high thiamine retention during frying agrees very well with earlier work (Waisman and Elvehjem, '41). More thiamine was retained after frying than after roasting, braising, or broiling (McIntire et al., '43).

Considerable amounts of each of the vitamins were found in the drippings from roasting and frying, an average of 10% of the thiamine and riboflavin, and 15% of the nicotinic acid.

The over-all retention from fresh stored to cured fried samples agrees very well with retention during curing and frying. Seventy-three per cent of the thiamine was retained during curing and an average of 86% during frying. This

compares with an average of 59% over-all retention as shown in table 3. Eighty-four per cent of the nicotinic acid was retained during curing and an average of 85% after frying, as compared to 72% over-all retention. Similarly 92% of the riboflavin was retained during curing and an average of 77% after frying in comparison to 74% over-all retention.

SUMMARY

1. The thiamine, nicotinic acid and riboflavin contents of fresh, fresh stored, and cured hams before and after cooking were determined.

2. Average retention during storage is 92% for the thiamine and nicotinic acid and 85% for the riboflavin.

3. The retention in curing was found to be 73% for the thiamine, 84% for the nicotinic acid and 92% for the riboflavin.

4. The average retention in the meat alone after roasting was 58% for thiamine, 79% for the nicotinic acid, and 74% for the riboflavin; after frying, 86% for the thiamine, 85% for the nicotinic acid and 77% for the riboflavin.

5. The average total retention in the meat plus drippings after roasting was 70% for thiamine, 96% for the nicotinic acid, and 84% for the riboflavin; after frying, 92% for the thiamine, 96% for the nicotinic acid, and 86% for the riboflavin.

6. The over-all retention of the vitamins from fresh stored to cured fried samples agrees very well with the vitamin retention during curing and frying.

7. From 10–15% of each of the vitamins was found in the drippings from roasting and frying.

8. A higher retention of thiamine in the meat alone was found after frying, as compared to roasting, braising and broiling.

The authors wish to express their thanks to Dr. Catherine J. Personius, Professor of Home Economics, for cooking the meats, and to Mr. Robert W. Bray of the Animal Husbandry Department for cutting the meats.

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STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM IN THE CHICK

I. THE COMPARATIVE EFFECT OF VITAMINS D_2 AND D_3 AND DIHYDROTACHYSTEROL GIVEN ORALLY AND INTRAMUSCULARLY

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(Received for publication January 20, 1943)

It was first noted by Hess and Supplee ('30) that irradiated ergosterol is much less effective than vitamin D from natural sources in promoting normal calcification in the chick. These observations have been amply confirmed, but no satisfactory explanation of the phenomenon has been found. Recently, Correll and Wise ('42) have shown that 1 antirachitic rat unit of dihydrotachysterol is equivalent in the chick to between 4 and 5 units of the U.S.P. Reference Oil. Their results have been confirmed by this laboratory, although we find that the exact ratio of effectiveness varies with the dose level. (A similar relationship of dose level to ratio of effectiveness for the chick has been shown to exist between vitamin D_2 and natural vitamin D by Massengale and Bills ('36).) Our experimental results are given in table 1. They were obtained by the A. O. A. C. vitamin D assay method for chickens with respect to diets and evaluation, except that the vitamin supplements were administered by stomach tube in corn oil, rather than in the diet.

Summarizing previous findings, then, there exists this peculiar situation: Vitamin D_2 is equivalent in the rat to

40,000 U.S.P. units per milligram [1] but in the chicken only to about 1,000 U.S.P. units; [2] vitamin D_3 is equivalent to 40,000 U.S.P. units per milligram in both rat and chicken; and crystalline dihydrotachysterol is equivalent to 80 U.S.P. units per milligram in the rat but to about 360 U.S.P. units in the chicken.

Thus, of two closely related products, both derived from ergosterol, one is more effective antirachitically in the rat than in the chicken, while the other is more effective in the chicken than in the rat. It is perhaps worth calling attention to the fact that equal amounts by weight of vitamin D_2 and dihydrotachysterol have quantitatively rather similar effects in the prevention of rickets in the chicken, while vitamin D_3 behaves in a manner strikingly different from either of these substances.

Beyond mere speculation, the only attempts thus far made to explain the low effectiveness of vitamin D_2 in the chick appear to have been those of Russell and co-workers ('31, '32, '34). They have shown that the amounts of vitamin D_2 and natural vitamin D recoverable from the feces of the chick are quite comparable (26.5 and 34.1% of the dose administered, respectively). It was not definitely settled whether any of the remainder of the dose was destroyed in the digestive tract. However, they showed that cod liver oil was effective in the chick when given either by mouth or intraperitoneally, while six times as many units of vitamin D_2 given intraperitoneally in oil failed to promote equivalent calcification. From these results, they concluded that the low effectiveness of D_2 in the chicken could not be attributed to failure of absorption from, or destruction in, the digestive tract. However, the oils they administered intraperitoneally were not satisfactorily ab-

[1] The most recent authentic information indicates that this value is 40,000 (Coward, '40–'41). However, information at hand in this and other American laboratories indicates that both vitamins D_2 and D_3 may be equivalent to about 50,000 U.S.P. units per milligram.

[2] This value was taken as a convenient approximation. It has been variously reported anywhere from 100 to 2000, but usually from 800 to 1300. For a review of the literature on this point see Remp and Marshall ('38).

sorbed and it is questionable whether the vitamins were utilized quantitatively.

The purpose of the work reported here has been to study comparatively the effects of vitamins D_2 and D_3 and dihydrotachysterol on the calcium and phosphorus metabolism of the chick with the eventual objective of determining so far as possible the reasons for the low effectiveness of D_2. Although

TABLE 1

Antirachitic effect of dihydrotachysterol in the chick.

GROUP NO.	MEDICATION, SUBSTANCE	DOSE		AVERAGE INDEX OF RECALCIFICATION [1]	AVERAGE PER CENT BONE ASH	VITAMIN D UNITS (CALCULATED FROM BONE ASH)	VITAMIN D UNITS EQUIVALENT TO 1 U.S.P. UNIT
		U.S.P. units	Micrograms				
1	None	0		—2.0	28.69		
2	Reference oil	1	8696	+0.64	34.26		
3	Reference oil	4	34784	+3.30	44.23		
4	Dihydrotachysterol	0.25	3.12	+1.28	37.71	2.05	8.2
5	Dihydrotachysterol	0.50	6.25	+2.37	39.31	2.50	5.0
6	Dihydrotachysterol	0.75	9.37	+2.90	41.68	3.25	4.3
7	Dihydrotachysterol	1.0	12.5	+3.58	45.59	4.75 (approximately)	4.75
8	Dihydrotachysterol	2.0	25.0	+3.18	45.02	4.5 (approximately)	2.25
Average							4.9

[1] For method of determination, see McChesney and Homburger ('40).

the response of the chicken to dihydrotachysterol is rather surprising, it cannot be stated whether the effect of this substance in the chicken *or* the rat should be regarded as anomalous until further information is available.

OUTLINE OF TEST METHODS

White Leghorn chicks of both sexes were received in the laboratory on August 26, 1942, on the second day of life. They were immediately offered water and our regular chick ration,

which is slightly modified from that of Hart, Kline and Keenan ('31); i.e., we substitute two parts corn oil for two parts yellow corn. From this point on the chicks were managed as recommended by Massengale and Bills. On the sixth day of life the birds were divided as to size into eighteen groups of ten chicks each, at which time the average weight approximated 62 gm. The depletion period continued until the seventeenth day of life when medications were begun: all food consumed was weighed, and the excreta were collected for weekly periods. Certain groups of chicks were sacrificed at weekly intervals, as shown in table 2. At the end of the regular 4-week test period (forty-fifth day of life), all the remaining groups were sacrificed. Unless otherwise indicated all the birds in the groups survived the test period.

The birds were killed by decapitation and the blood was collected in centrifuge tubes from individual animals. The serum was separated by centrifugation, then pooled samples, consisting of 1 cc. of serum from each bird in the group, were analyzed for calcium and phosphorus. Calcium was determined by the Clark and Collip procedure ('25), and phosphorus by the method of Fiske and Subbarow ('25). The left tibia was removed from each bird and prepared for ash determination as described by Massengale and Bills.

Food and feces were wet ashed in a mixture of nitric and sulfuric acids. On the solution thus obtained, calcium was determined by the procedure of Shohl and Pedley ('22). Phosphorus was determined by the method of Fiske and Subbarow, adjusting the concentration of sulfuric acid so that the sample taken for analysis was contained in 1 cc. of 2 N H_2SO_4. The feces, with some spilled food admixed, were dried, weighed, finely ground in a corn mill, and thoroughly mixed by shaking. Samples of 2.50 gm. were taken for ashing, and the appropriate aliquots of the ash solution taken for analysis. The food was analyzed in the same way and was found to contain 0.90% calcium and 0.70% phosphorus. During the test period the chicks received distilled water ad libitum, changed daily.

Calcium and phosphorus balances were calculated by subtracting the total output in milligrams for the week from the total intake, and dividing by the number of chick-days (i.e., usually 70, but less in case one or more chicks died). Balances are, therefore, recorded in terms of milligrams per chick per day.

Medications were given according to the following plan: oral medications (groups 1 to 12) were given by direct injection by tube into the crop. The vehicle used was corn oil. Quantities of 0.25 cc. were given on the first day of the weekly periods, and quantities of 0.50 cc. were given on the second, fourth and sixth days — or a total weekly volume of 1.75 cc. The intramuscular injections (groups 13 to 17) were given bi-weekly in a volume of 0.1 cc. at each injection. The compounds were dissolved in either corn or sesame oil, or propylene glycol. The injection was made deep into the breast muscle, adjacent to the sternum.

The dosages selected for oral administration were chosen on the basis of the following reasoning. Approximately 3 units of vitamin D_3 per day by the oral route are required to promote entirely normal calcification in the chick over a 28-day period (see McChesney and Homburger, '40). Vitamin D_2 was given in a dosage of 120 units per day on the basis that the effectiveness of D_2 is generally accepted at about one-fortieth that of vitamin D_3. Dihydrotachysterol was administered in a dosage of only 0.6 rat unit per day since this should be equivalent in the chick to about 3 units of D_3, as previously explained (Correll and Wise, '42).

Vitamin D_3 was given intramuscularly in oil at a level of 96 units per week, or about 4.6 times the oral dose. Such a dosage ratio appeared logical, since the observations of Barlow and Kocher ('42) indicated that vitamin A in oil is only 10 to 15% as effective intramuscularly as when the same dose is given by mouth. The work of Liebe ('39) also has shown that in order to produce equal degrees of calcification, the rat requires about ten times as much vitamin D in the form of Vigantol by the parenteral route as is necessary when

given orally in the same medium. Vitamin D_3 in propylene glycol was given intramuscularly at a level of 20 units per week, since Barlow and Kocher have shown that vitamin A in this menstruum is as efficiently utilized whether given intramuscularly or orally. The glycol is miscible with the tissue fluids and therefore does not remain in situ for long periods of time as does oil.

Vitamin D_2 in propylene glycol was also administered intramuscularly in a dosage of 20 units per week in order to compare the effects of an exactly equal number of U.S.P. units of D_2 and D_3 on calcium and phosphorus metabolism when given by this route. Since we can be positive that the solvent used for each form of the vitamin is absorbed, any differences observed in response to this therapy can only be attributed to the specific differences in the manner in which they affect the mineral metabolism of the chick in that any possible difference through destruction and poor absorption from the gastrointestinal tract is ruled out by these procedures. It was for the same purpose that we administered both vitamins D_2 and D_3 intramuscularly in oil, at a level of 96 units per week, thus permitting an exact comparison of these compounds in oil.

Finally, dihydrotachysterol was administered intramuscularly in oil in a dosage equivalent to 22 U.S.P. units per week. This ratio of intramuscular to oral dosage corresponds to that previously selected for the intramuscular oily injections of D_2 and D_3. It was our purpose throughout this experiment to give marginal or preferably suboptimal vitamin dosages since it is under these conditions, rather than with excessive dosages, that comparisons are best made.

RESULTS

The numerical data are given in table 2.

Discussion of results

Growth. The birds treated with the oral supplements of vitamins D_2 and D_3, as well as those which received vitamin

D_3 intramuscularly, grew at a very satisfactory rate. The birds which received dihydrotachysterol showed somewhat retarded growth: those receiving 4.2 rat units (R.U.) per week orally gained only 95% in weight during the 4-week test period as against a gain of 141% for the chicks which received 21 U.S.P. units per week of vitamin D_3. The chicks which received 22 R.U. of dihydrotachysterol intramuscularly showed a still greater retardation of growth in that a body weight of only 60% occurred during the test period. These birds were in a very poor condition and some were unable to survive. This restriction of growth on dihydrotachysterol was also noted by Correll and Wise, although not until they had given oral dosages of the order of 25 units per week (equivalent to about 400 units intramuscularly).[3] They attributed the poor growth to toxicity. It is, of course, possible that the preparation used in this work was more toxic than that used by Correll and Wise. It was not the same one as we used in the test reported in table 1, in which all groups grew satisfactorily. Our laboratory data indicate little difference between the two preparations: they are antirachitically equal in potency and the preparation used in the work reported in table 2 is at most twice as toxic for mice as the one used in the test reported in table 1. For the description of the method used in determining toxicity, see McChesney and Kocher ('42).

The most unexpected response in connection with growth is that of the group which received 96 units of vitamin D_2 intramuscularly per week in corn oil (group 13). These birds were in much worse condition than the negative controls and gained only 41% in weight during the test period. The chickens showed loss of appetite, were extremely weak and unsteady on their feet, and there were many deaths, possibly from malnutrition. It appears that vitamin D_2 given by the intramuscular route in an insufficient dose, that is, 20%

[3] McChesney, E. W., unpublished data.

TABLE 2

Effects of various activated sterols on the calcium-phosphorus metabolism of the chick.

GROUP NO.	AGE IN DAYS	AVERAGE WEIGHT, GRAMS	MINERAL BALANCE, MG. PER CHICK PER DAY		BLOOD SERUM MG. PER 100 ML.		BONE ASH PER CENT	VITAMIN SUPPLEMENT PER CHICK PER WEEK
			Calcium	Phosphorus	Calcium	Phosphorus		
1	17	103						
	24	132	53.3 [1]	31.9 [1]	8.4	5.4	35.05	
2	17	105						
	24 [2]	144	38.9	22.8				
	31	182	59.3	45.7	8.6	5.6	35.96	
3	17	107						Vitamin D_2 in corn oil, 840 units orally
	24	130	46.1	29.6				
	31	170	70.3	51.6				
	38	204	60.9	42.3	9.0	4.8	38.22	
4	17	106						
	24	126	54.0	35.3				
	31	161	52.4	43.1				
	38	215	77.3	51.3				
	45	262	77.4	51.7	9.2	5.3	40.50	
	Group average [3]		60.0	45.0				
5	17	107						
	24	128	44.1	32.0	8.7	5.6	33.95	
6	17	107						
	24	136	47.4	25.6				
	31	169	54.7	36.9	8.9	5.9	37.77	
7	17	111						Vitamin D_2 in corn oil, 21 units orally
	24	138	55.4	35.6				
	31	171	43.0	27.7				
	38	235	78.1	51.7	9.3	5.9	39.13	
8	17	106						
	24	127	42.6	26.0				
	31	170	46.7	29.9				
	38	209	86.7	63.7				
	45	255	78.0	51.3	9.2	5.5	39.63	
	Group average [3]		63.5	42.5				
9	17	111						
	24	138	54.1	33.6	7.7	5.2	32.43	
10	17	112						
	24	134	46.4	27.7				
	31	162	36.4	26.1	8.7	5.4	34.48	
11	17	107						Dihydrotachysterol in corn oil, 4.2 units [4] orally
	24	132	49.6	32.0				
	31	160	42.0	24.0				
	38	184	44.1	30.9	8.0	5.2	36.79	
12	17	108						
	24	130	40.4	24.0				
	31	158	43.9	29.9				
	38	183	44.7	23.7				
	45	211	45.3	24.7	8.6	4.9	38.17	
	Group average [3]		44.0	25.5				

TABLE 2 (Continued)

GROUP NO.	AGE IN DAYS	AVERAGE WEIGHT, GRAMS	MINERAL BALANCE, MG. PER CHICK PER DAY		BLOOD SERUM MG. PER 100 ML.		BONE ASH PER CENT	VITAMIN SUPPLEMENT PER CHICK PER WEEK
			Calcium	Phosphorus	Calcium	Phosphorus		
13	17	107						
	24	122	20.6	12.5				
	31	129	17.8	12.3				Vitamin D_3 in corn oil, 96 units intramuscularly
	38	139	15.1	8.7				
	45	155 [5]	13.2	10.0	6.4	6.1	31.42	
		Average	16.8	11.0				
14	17	107						
	24	134	25.9	16.7				
	31	159	17.7	14.7				Vitamin D_3 in propylene glycol, 20 units intramuscularly
	38	180	38.0	28.0				
	45	184	25.4	17.3	6.6	6.0	33.68	
		Average	26.8	19.2				
15	17	106						
	24	139	27.7	21.3				
	31	169 [6]	32.4	29.9				Vitamin D_3 in corn oil, 96 units intramuscularly
	38	212	68.3	44.4				
	45	244	63.0	37.6	9.8	6.3	38.53	
		Average	47.8	33.2				
16	17	106						
	24	137	45.9	28.6				
	31	153	78.4	45.6				Vitamin D_3 in propylene glycol, 20 units intramuscularly
	38	220	108.9	61.6				
	45	308	100.0	62.1	10.0	6.5	45.57	
		Average	83.2	49.5				
17	17	108						
	24	124	27.3	20.0				
	31	135	21.7	18.0				Dihydrotachysterol in sesame oil,[7] 22 units [4] intramuscularly
	38	157	23.4	15.5				
	45	173 [8]	23.0	13.1	9.1	4.7	34.93	
		Average	23.8	16.8				
18	17	103						
	24	121	27.0	21.1				
	31	139	22.6	15.5				
	38	150	21.9	14.1				None
	45	158 [9]	19.1	14.3	7.5	5.2	30.39	
		Average	22.6	16.2				

[1] Balances given are daily averages for the weekly period between the two ages mentioned; i.e., from the 17th to the 24th day of life, and for the period from the 24th to the 31st day of life, etc.

[2] One chick died on the 22nd day of life.

[3] These averages refer only to groups 4, 8 and 12.

[4] In this paper a U.S.P. rat unit refers to the antirachitic effect of 1 unit of U.S.P. reference oil in the albino rat. In terms of the crystalline preparations referred to it would be: vitamins D_2 and D_3, 0.025 $\mu g.$; dihydrotachysterol, 12.5 $\mu g.$ In the chick a U.S.P. unit would be approximately: vitamin D_2, 1 $\mu g.$; vitamin D_3, 0.025 $\mu g.$; dihydrotachysterol, 2.8 $\mu g.$

[5] Two chicks died on the 23rd day of life, one on the 35th, one on the 39th, two on the 43rd and one on the 45th.

[6] One chick "missing" on the 28th day of life; presumably died.

[7] Sold by the Winthrop Chemical Company under the trade name, "Hytakerol." One ml. contains 1.25–1.50 mg. dihydrotachysterol and assays 100–120 U.S.P. rat units.

[8] One chick died on the 33rd day of life, one on the 36th, and one on the 43rd.

[9] One chick died on the 44th day of life.

or less of the curative dose, weakens rather than strengthens the chicks. Further study will be required to account for this reaction. Those chicks which received 20 U.S.P. units of vitamin D_2 weekly on propylene glycol also did not grow very well, but they grew better than the negative controls, and as well as the birds which received dihydrotachysterol by mouth.

Mineral balances. The results indicate that the amount of mineral retention does not necessarily increase with each week of life and that under oral dihydrotachysterol medication the retentions are practically uniform for each week as contrasted with vitamins D_2 and D_3 where they generally increase each week. The effects of the last two named were quite comparable, and the response to medication was immediate.

The birds which received vitamin D_2 intramuscularly in oil showed balances much poorer than the negative controls. However, those which received the vitamin intramuscularly in propylene glycol showed balances slightly greater than those of the negative controls. The data indicate further that vitamin D_3 given intramuscularly in oil produces no immediate effect on calcification. By the third week the birds began to retain amounts of calcium and phosphorus almost equal to those observed in the animals which received the vitamin orally. The results of the analyses indicate that the vitamin is very slowly or poorly utilized when administered in oil by the intramuscular route. The birds which received the vitamin D_3 intramuscularly in propylene glycol made the most satisfactory showing of all the groups tested. The birds which received dihydrotachysterol intramuscularly in oil had mineral balances almost exactly the same as those of the negative controls.

Blood levels. Although no control figures were obtained for the birds at the start of the test period, it is apparent that the values for calcium at least were low. The most prompt recovery was noted in the birds which received vitamin D_3 orally, the values for both calcium and phosphorus being only slightly below normal at the first observation, and improving

steadily thereafter. The changes in phosphorus from week to week hardly seem significant as no trend is evident. The birds which received vitamin D_2 orally showed improvement in calcium levels for each week, but the values obtained were always slightly lower than those in the D_3 chicks until the last week. There is no apparent reason for the low phosphorus value for the third week.

The chicks which received dihydrotachysterol orally gave particularly low calcium levels for the first and third weeks and the values were, in fact, always at a lower level than in the D_2 or D_3 birds. However, the phosphorus values for these birds did not differ significantly from those of the other groups mentioned.

As for the blood levels of the birds which received the intramuscular injections, those which received vitamin D_3 in either solvent showed very satisfactory blood levels, indicating an entirely normal state of calcium and phosphorus metabolism at the end of the test period. The negative controls showed low calcium, but normal phosphorus values. The birds which received vitamin D_2 in either solvent showed deranged metabolism as indicated by very low serum calcium. The values obtained for the birds which received dihydrotachysterol were at the lower limits of normal.

Bone ash. The bone ash percentages are somewhat lower than those obtained in the usual chick D assay, because of the rather long depletion period. Nevertheless, the oral vitamin supplements were sufficient in every case to permit the birds to show an increased ash value for each week of the test period.

The bone ash values in general agree with what would have been expected from the observed mineral balances. There are some exceptions to this, notably in group 9 where a rather high balance is accompanied by a low ash. Otherwise, with respect to groups killed at the same time there is a good correlation between mineral balances and bone ash.

Relative effectiveness of preparations. The results obtained make possible some tentative conclusions as to the relative effectiveness of the various preparations tested. Since vita-

min D_2 was administered orally at a level of 40 units for each unit of vitamin D_3, and gave a slightly superior result, it is evident that in these experiments the previously reported ratio of effectiveness of about 35 to 1 is confirmed. Dihydrotachysterol was administered orally at a level of 1 unit for each 5 units of vitamin D_3 and it gave a slightly inferior result (as regards bone ash); therefore in these experiments and at this particular dose level, the relative effectiveness must have been approximately 4 units of D_3 equals 1 unit of dihydrotachysterol. Because of the lack of points on the dose-response curve, a more exact quantitative comparison is not possible, but this will be the subject of a subsequent publication.

Vitamin D_2, administered intramuscularly in either oil or propylene glycol in the same unit doses as vitamin D_3, exerted a much inferior antirachitic effect. Quantitative comparison of the two vitamins is not possible. The same applies to dihydrotachysterol in oil. However, the difference between groups 15 and 17 indicates plainly that dihydrotachysterol in oil by muscle is considerably less than four times as effective as vitamin D_3 per rat unit by the same route.

Vitamin D_3 given at a level of 20 units per week in propylene glycol intramuscularly is much more effective than 21 units orally in oil, indicating that there is a serious loss in absorption (probably at least 50%) and that by the intramuscular route the vitamin must be as completely utilized as can be accomplished by any route of administration.

CONCLUSIONS

1. The ratios of oral effectiveness in the chick of $D_3 : D_2 = 35 : 1$, and of dihydrotachysterol : D_3 = about 4.5 : 1 (rat unit for rat unit) have been confirmed.

2. Vitamin D_2, given intramuscularly in either oil or propylene glycol, and at the same unit dose level as vitamin D_3, has a much inferior effect and the mineral balances of the chicks are very low.

3. Dihydrotachysterol administered intramuscularly in oil is not as effective relative to vitamin D_3 as when the two preparations are administered orally.

4. Vitamins D_2 and D_3, administered orally in doses equivalent in antirachitic effect (for the chick), maintained normal calcium and phosphorus metabolism. However, a dosage of dihydrotachysterol equivalent in antirachitic effect to these does not serve to maintain normal serum calcium levels and the mineral retentions are less.

5. Vitamin D_2 and dihydrotachysterol, administered intramuscularly in small doses in oil, definitely weaken the chicks.

6. Vitamin D_3 administered intramuscularly in propylene glycol is much better utilized than when given orally in oil. When given intramuscularly in oil it is much more poorly utilized than when given orally.

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VITAMIN K AND PROTHROMBIN LEVELS WITH SPECIAL REFERENCE TO THE INFLUENCE OF AGE [1]

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TWO FIGURES

(Received for publication January 18, 1943)

It is now firmly established that vitamin K is concerned in the production of prothrombin, but the mode of its action remains obscure. Investigation of this mechanism from the metabolic standpoint immediately raises the problem of exact quantitative relationship between the vitamin K intake and the prothrombin level, and of factors which may influence this quantitative relationship. Among the more obvious of such factors are the age and rate of growth of the animal, tissue storage of the vitamin and of prothrombin, and the functional integrity of the tissues concerned in the production and utilization of prothrombin.

A certain amount of more or less incidental information of a quantitative nature has been obtained in connection with various assay procedures for vitamin K, but unfortunately, most of the assay procedures have been based simply upon hemorrhagic symptoms, or upon crude clotting time tests. The data are therefore not quantitative with respect to prothrombin levels. In the clinical studies on man, more careful attention has been given to the prothrombin level, but the patient's welfare usually has dictated the use of large amounts

[1] Aided by a grant from the John and Mary R. Markle Foundation. Funds for technical assistance were also provided by the Graduate College, State University of Iowa.

of the vitamin, and very little is known about the response to suboptimal dosage.

Of the data available, perhaps the most significant from the metabolic standpoint is some of the vitamin K assay work of Almquist, Mecchi and Klose ('38), and of Almquist and Klose ('39). These investigators reported that in the chick the reciprocal of the clotting time or the prothrombin time approximates a straight line function of the logarithm of the vitamin K level in the diet. The relationship is purely empiric, and is not reliable for minor degrees of deficiency. Their work was carried out with vitamin K concentrates rather than with some of the pure synthetic preparations now available, and the prothrombin level was determined for only a single interval for each individual chick. Furthermore, the active principle was given ad libitum, by adding it directly to the diet, and there is no precise way of knowing either how much was eaten, or how much may have been lost by deterioration. It is particularly difficult to compare the amount of vitamin actually consumed by chicks of different ages when the vitamin is administered in this way. It was therefore thought desirable to establish more exact baselines for metabolic work, with elimination of these various uncertainties, in so far as possible. In the work reported at this time we have employed the two-stage prothrombin method which, in our experience, is more precise than the one-stage procedures for estimating the prothrombin level. Also, the one-stage tests do not show a difference between the prothrombin of the young and of the adult, whereas with the two-stage method, the prothrombin of the chick, like that of other species, is shown to be low normally at hatching and to gradually rise to the adult level over a period of several months (Tidrick, Joyce and Smith, '39).

EXPERIMENTAL

Newly-hatched White Leghorn chicks were placed on a slightly modified Almquist's diet, as described in a previous publication (Tidrick, Joyce and Smith, '39). The animals were kept on raised wire screens to minimize coprophagy and a

constant source of running water was provided to prevent contamination of the drinking water. Blood for prothrombin determination was drawn into potassium oxalate solution from the heart or from the jugular vein. The blood was centrifugalized, and the assays made on the plasma by the two-stage method of Warner, Brinkhous and Smith ('36), except that chick brain instead of beef lung was used as the source of thromboplastin. The daily dose of vitamin K (2-methyl-1, 4-naphthoquinone), dissolved in 0.2 cc. of corn oil [2], was introduced directly into the crop with the aid of a syringe and metal tube.

The normal control group of 198 chicks received a diet which was rich in natural vitamin K, but, to ensure an abundance of the vitamin, each chick was given a supplement of 12.5 μg. of the synthetic vitamin daily by injection directly into the crop. The test animals, approximately 300 chicks, were placed on the deficient diet and given varying doses of the vitamin. They were about equally divided among the various vitamin K dosage levels. A part of them were given the vitamin supplements from the time of hatching; the remainder were given no vitamin for the first 10 days of life and were then given restorative doses varying in size. By the age of 2 weeks, the prothrombin level had become stabilized for each vitamin K dosage and the chicks given no vitamin for the first 10 days had reached essentially the same levels as those given the vitamin supplements from the time of hatching.

RESULTS AND DISCUSSION

In figure 1, the data are plotted to show the relation between the vitamin K intake and the prothrombin level after stabilization had been reached. The average prothrombin level for each dosage at the age of 17 days is plotted in the solid columns. It is evident that as maintenance requirements were approached a given increment in the amount of vitamin administered had somewhat less effect. Amounts greater than 2 μg. per day did not produce any further elevation of the

[2] Mazola.

prothrombin level and the shape of the interrupted curve would indicate that the minimal requirement cannot be much less than this amount.

In the continuous curve, we have plotted hypothetical prothrombin values, obtained by calculating the prothrombin level as a simple function of the logarithm of the vitamin K intake, as suggested by the work of Almquist et al., mentioned above. With large doses of the vitamin, i.e., larger than 2 μg., the logarithmic relation could not be expected to hold; they were

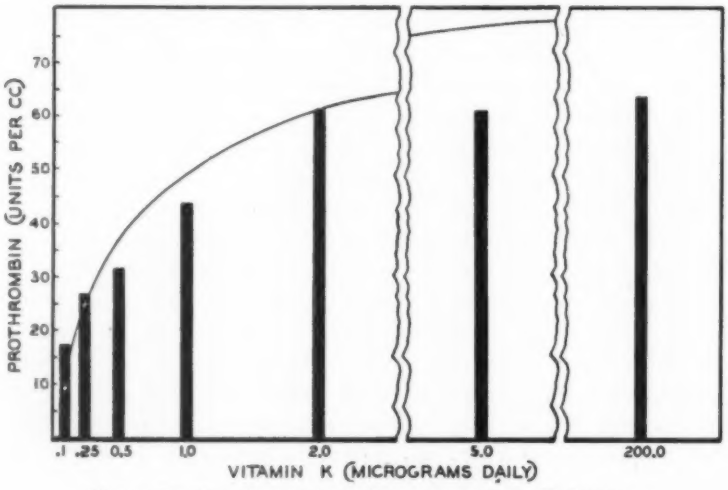

Fig. 1 Prothrombin levels with various vitamin K dosages.

therefore not given consideration in determining the constants for the logarithmic equation. Also, the lowermost observation (0.1 μg.) deviates rather markedly from the hypothetical curve and this value was given less than its full weight in determining the constants which gave the best fit with the experimental data. It may be that the prothrombin level obtained with 0.1 μg. per day is too high. Minor degrees of contamination as a result of coprophagy, or residual vitamin in the diet, would be expected to result in a conspicuous error at this low dosage level. Even with these limitations, it can be seen that

the approximation of the hypothetical values to the actual prothrombin levels obtained experimentally is not very exact. It is of interest that the animals maintained on the extracted diet did not attain quite as high prothrombin values as did the normal controls, even though massive doses of the vitamin were given. Thus, those given 200 μg. per day reached a level of 62 units whereas the control group, as shown below, reached a level of 68 units per cubic centimeter at this same age. This difference might be interpreted to mean that the synthetic

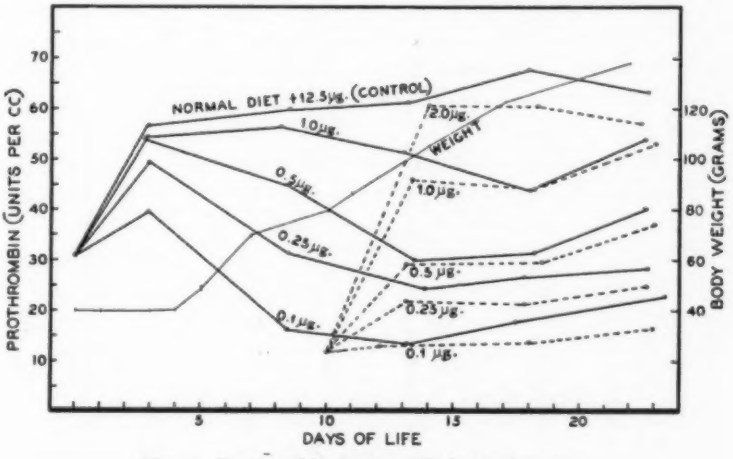

Fig. 2 Prothrombin levels with increasing age.

material was not as effective as the natural vitamin K present in the unextracted diet. It seems more likely, however, that the normal control diet (brewers' yeast 8.0%, fish meal 17.0%, polished rice 61.5%, alfalfa meal 10.0%, salt mixture 2.5% and cod liver oil 1.0%) contains some other essential factor, and that a deficiency of this factor interferes with completely normal prothrombin production.

In figure 2, the data are plotted to show the influence of age and size of the chick on the prothrombin level with varying amounts of vitamin K. The animals receiving the vitamin from the time of hatching are indicated by the continuous

curves; those given no vitamin for the first 10 days, by the broken lines. For simplicity in charting, the experimental period was divided into 5-day intervals, the average prothrombin level and the average age of the chicks when tested being determined for each 5-day period.

During the first few days of life the prothrombin level rose rapidly above the value at hatching of approximately 31 units per cubic centimeter of plasma. The rise was greatest in the normal control animals given an enriched diet with a supplement of 12.5 μg. of the vitamin per day. The low hatching level shown in the chart does not appear, however, to be due to a shortage of vitamin K, for similar values have been obtained even though large amounts of the synthetic vitamin were administered to the laying hens (unpublished data). Also, by injection of the vitamin directly into the incubating eggs, the newly-hatched chicks can be shown to have excess stores of the vitamin (Tidrick, Stamler, Joyce and Warner, '41), and despite the presence of such stores, the hatching level remains low. Furthermore, there was a similar initial rise in prothrombin in the chicks given minimal amounts of the vitamin. Even in the chicks given no vitamin whatever (not shown in the figure) there was a definite rise in prothrombin level during the first few days. This initial rise may be due to increased activity of tissues which are becoming more mature. On the other hand, it seems equally possible that the hatching level is pathologically low as a result of some deficiency other than vitamin K, and that this deficiency is rapidly corrected when the chick begins to eat. Some such hypothetical deficiency is suggested by the fact that we occasionally obtain a batch of eggs in which the hatching level is as high as 50 units per cubic centimeter. This would seem to imply that the hatching level of 31 units per cubic centimeter shown in the figure is not necessarily normal. In our experience, eggs with an unusually high hatching level are more often obtained in the summer and early fall months, when a widely varied diet is available to the hens.

After the initial rise, the prothrombin becomes more or less stabilized at a level determined by the amount of vitamin K administered. With the lower dosages of the vitamin, this stabilization was at a level much lower than that present at the time of hatching. The chicks given no vitamin for the first 10 days of life were started on supplements of the various dosages at this time in order to approach the prothrombin level attained for each dose from the opposite direction. By the age of 15 days these animals, shown in the broken lines on the chart, reached essentially the same levels as obtained in the animals given the vitamin from the time of hatching, though there was a slight tendency to lag on the part of the animals depleted for the first 10 days.

In all of this work there was considerable variation in the prothrombin values from chick to chick, with some actual overlapping for the different dosage levels. The differences between the average prothrombin levels for each group, however, are consistent throughout the experiment, and at most points exceed the probable error of the difference severalfold. At the age of 17 days, for example, the observed difference in the average prothrombin values for the 0.5 μg. and the 1.0 μg. groups receiving the vitamin from the time of hatching, is three and one-third times the probable error of that difference.

Following stabilization, the prothrombin level for each dosage tended to rise gradually throughout the remainder of the experiment, despite the fact that the vitamin was kept constant. Even in the groups given only 0.1 μg. per day — an amount barely sufficient to prevent death from hemorrhage during the first 2 weeks of life — there was a definite tendency for the prothrombin level to rise slightly as the chick grew older. Data for older chicks are not as complete as for the age period shown in the chart. We have, however, obtained sufficient data to indicate that the prothrombin level continues to rise slowly during the next 2 months. This is somewhat surprising when one considers that the chick at 3 weeks of age has trebled in weight, and at the end of 2 months weighs nearly fifteen times the hatching weight.

It is of course possible that traces of vitamin K remained in the extracted diet and that, with increasing food intake, the older chicks obtained increasing amounts of this contaminating vitamin. Both the brewers' yeast and the fish meal used in the diet, however, were subjected to continuous ether extraction for 10 days in a giant Soxhlet apparatus. The cod liver oil was used in only small amounts (1%) and no vitamin K could be demonstrated in the preparation used by giving large amounts of the oil to test chicks. Thus, it seems unlikely that appreciable amounts of vitamin K could have remained in the diet. As a second possible source of contamination, greater production of vitamin K by bacteria in the intestine could conceivably supply additional vitamin as the chicks increased in age. That these are not important sources of vitamin K in these experiments, however, is indicated by the fact that minute increments in the vitamin K supplement continued to have a marked effect on the prothrombin level. Were the chicks obtaining significant amounts of the vitamin from incomplete extraction of the diet, or from bacterial synthesis in the intestine, one would hardly expect the groups on slightly different dosage levels to continue to have significantly different prothrombin levels.

It is evident that the vitamin K requirement of the chick is not related in any simple way to the body weight, or surface area. The requirement seems more nearly analogous to the decreasing need for pantothenic acid in the growing rat (Unna and Richards, '42), or to the iron requirement of the growing child (Heath and Patek, '37). The lack of need for additional vitamin K as the animal increases in size might be explained on the basis of progressively greater efficiency of utilization. On the other hand, it may well be that the amount of vitamin required for maintenance is minimal. If so, one might postulate that the vitamin K which is required is needed mainly for growth. There is very little evidence, however, that the vitamin is needed for increase in size itself, for the animals grow at the normal rate even when a severe deficiency exists. The need of the growing organism for relatively large rations of

vitamin K may represent merely the needs for building new prothrombin to correspond to the increase in body size and blood volume.

SUMMARY

Young White Leghorn chicks require 1.0 to 2.0 μg. of 2-methyl-1, 4-naphthoquinone per day to maintain the prothrombin at a normal level. Only about one-twentieth of this amount, however, is necessary to maintain sufficient prothrombin in the blood to protect the animal from hemorrhagic manifestations. Thus, there is a wide range between the amount of vitamin which will support normal prothrombin levels and the amount necessary for protection from hemorrhage.

There is no increase in the amount of vitamin K needed by the young chick as the animal increases in age, despite the fact that the body weight increases severalfold within a few weeks. The data suggest that a large portion of the vitamin needed by the growing animal is associated with the increase in size.

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